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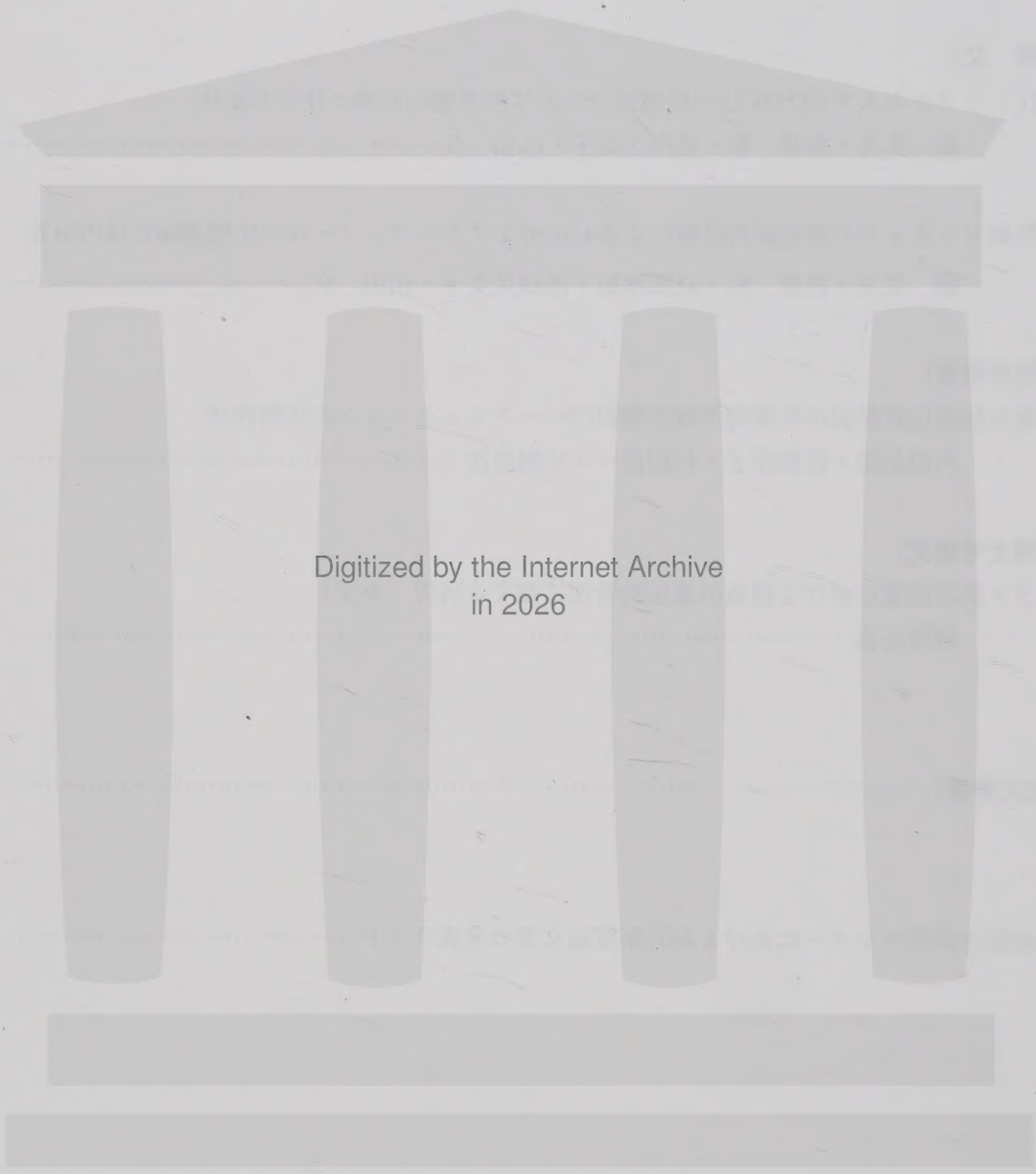

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塩化トリブチルスズ(TBTCl)の組織器官への生物濃縮の成熟に伴った変化

堀　英夫[*1]，角埜　彰[*2]，池田久美子[*2]，山田　久[*2]

Changes in bioaccumulation of tributyltin chloride (TBTCl) in the tissues and organs of immature and maturating mummichogs (*Fundulus heteroclitus*)

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Abstract Male and female mummichogs (*Fundulus heteroclitus*) were exposed to waterborne tributyltin chloride (TBTCl) for 56 or 77days at different maturation stages during the refractory and the maturating periods. Differences in TBT bioaccumulation in the tissues and organs as well as entire body by sex and degree of maturation were studied for a discussion on the influence of TBTCl on the reproduction processes.

The BCF during the refractory period was 9,100±640 for the males and 9,200±1,700 for the females, indicating that mummichogs bioaccumulated TBTCl in much higher concentration than other marine fish species. The TBT concentration was the highest in the blood and the lowest in the digestive tract of male and female mummichogs. The TBT concentrations in the liver and gonads were similar to one another. The differences between the males and females in terms of TBT concentration and BCF were neglegible during the refractory period. On the other hand, TBTCl had bioaccumulated significantly in the testes during the maturating period, and the BCF was 7 times larger in the testes than in the ovaries. The high bioaccumulation in the testes suggests that TBTCl inhibits the reproduction processes of male fish more seriously.

Key Words: BCF, tissue distribution, maturation, mummichog, tributyltin chloride

船底防汚塗料の殺生物剤として使用されてきたトリブチルスズ（TBT）化合物の魚類に対する有害性については，急性毒性の把握（小山，清水，1992；堀口，清水，1992），初期生活段階毒性試験による慢性毒性（清水，1992）および最大許容毒物濃度の把握（小山，清水，1992），無影響濃度の推定（小山，清水，1992），生物濃縮係数の把握（山田，1992; Yamada and Takayanagi, 1992），病理組織観察による標的臓器の推定（Wester *et al.*, 1990; 清水，木村，1992; 清水，木村，1994）などの多くの研究が行われてきた。これらの研究の成果から，TBT化合物は，魚類に高濃度に蓄積されるとともに，急性および慢性的な毒性を有することが明らかにされた。

酸化トリブチルスズ（TBTO）の海産魚の組織・器官に及ぼす長期的影響を調べた研究（清水，木村，1992; 小山，清水，1992; 清水，木村，1994）では，TBTOが①鰓上皮の肥厚および過形成，②肝細胞の萎縮，変性および壊死，③尿細管硝子滴変性，糸球体腔拡張および尿細管の壊死，④造血細胞の減少，⑤腸管上皮細胞の萎縮および壊死，⑥表皮の変性，萎縮およ

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び配列の乱れ，⑦卵巣の発達阻害，⑧胸腺の萎縮並びに⑨精巣の発達阻害および精子形成の停止を引き起こすことが報告された。①から⑦の組織・器官の異常が飼育水中TBT濃度が1.0～2.1 μg/L（96時間 LC_{50} 値の1/2～1/4）の比較的高濃度の場合に起こるのに対し，⑧胸腺の萎縮並びに⑨精巣の発達阻害および精子形成の停止は，飼育水中TBT濃度が0.1 μg/L（96時間 LC_{50} 値の約1/40）の低濃度において起こることが明らかにされた。したがって，TBT化合物の海産魚類に対する特徴的な作用は，胸腺組織を中心とした造血免疫系への影響および精子形成を中心として雄の生殖系への影響であると指摘されている（清水，木村，1994）。

TBTOは卵巣に比較して精巣に対し特に著しい影響を及ぼすことが明らかにされているが，生殖腺の発達阻害に対する作用機構はほとんど解明されていない。生殖腺に対する影響が雌雄で異なることは，TBT化合物の蓄積濃度が卵巣と精巣で異なることに起因することも考えられるが，その詳細は明らかでない。したがって，本研究ではTBT化合物の生殖腺に対する作用機構と生殖腺に対する影響の雌雄による差異の一端を解明するために，飼育水から暴露された塩化トリブチルスズ（TBTCl）の卵巣および精巣をはじめとする組織・器官への分布および生殖腺の成熟に伴った蓄積濃度の変化を明らかにすることを目的としている。

なお，本研究で試験魚として用いたマミチョグは北アメリカ大陸東岸汽水域に生息する広塩性の小型魚であり，小型水槽で容易に成熟させることができる。マミチョグの我が国における成熟産卵周期はShimizu（1997）により研究され，GSIの季節変化および繁殖時期が解明されている。したがって，小型水槽による実験の容易さの他に不応期および成熟開始時期からの暴露試験計画の立案が容易なために試験魚としてマミチョグを選定した。

材料と方法

塩化トリブチルスズ（TBTCl）

東京化成工業㈱製の試薬（純度95%）を暴露試験に用いた。暴露試験開始までは冷暗所に保存した。一方，関東化学㈱製の有機スズ化合物標準原液（1 mg/mLトルエン溶液）を化学分析用標準物質として使用した。また，代謝物質分析のためにAldrich社製の塩化モノブチルスズ（$MBTCl_3$）（純度95%）および塩化ジブチルスズ（$DBTCl_2$）（純度96%）を化学分析用標準物質として使用した。

不応期における暴露試験

試験生物

瀬戸内海区水産研究所で産卵・ふ化させた後に屋内水槽において自然水温で飼育したマミチョグ（*Fundulus heteroclitus*）成魚（雄および雌）を試験に用いた。試験は産卵が終了したマミチョグを用いて不応期にあたる10月31日～12月26日に行った。また，試験に用いた雌マミチョグの体長および体重は，それぞれ11.3±0.6cmおよび22.9±3.7gであった。また，雄マミチョグの体長は10.7±0.2cm，体重は19.4±2.2gであった。

暴露試験

雌暴露区および雄暴露区を設定し，堀ら（2002）が作成した循環濾過式生物濃縮試験装置を用いて試験を行った。角型ガラス製水槽（容積約58L）を試験水槽として用い，雌暴露区には15尾，雄暴露区には20尾収容した。30L/hの流量で飼育水を各水槽に供給し，水槽内の飼育水を1日に約15回換水した。

既報（堀ら，2002）の実験と同様な方法でTBTClを試験水槽へ導入した。すなわち，ジメチルスルホキシド（DMSO）に溶解したTBTCl原液を，撹拌混合させながらろ過海水に溶解させて中間薬液を調製した。この中間薬液を，微量定量ポンプ（Iwaki社製，EH-B10 S-100PR9-L型）を用いて一定流量で試験水槽に添加し，飼育水中のTBT濃度を一定に維持した。平成12年10月31日～12月26日の56日間暴露試験を実施した。

試験期間中は水温を20℃に保ち，室内の照明を16時間明，8時間暗の明暗周期で制御した。また，体重の2%の配合飼料（マルハ(株)製，マルハヒラメ稚魚1号）を1日2回（朝と夕方）に分けて投与した。毎朝残餌および糞を取り除いた。

暴露区試験の14日目，28日目，42日目および56日目にマミチョグを取り上げ，採血した後に解剖し，肝臓，生殖腺，消化管およびその他の部位（筋肉，心臓，鰓，骨，表皮等，以下「その他」と表記）に分け，それぞれの部位をひとまとめにした。2～3尾ずつひとまとめにして3個の分析用試料を調製した。さらに，「その他」から筋肉を一部採取し，これら採取した血液（全血），肝臓，生殖腺，消化管，「その他」および筋肉についてTBT濃度を測定した。「その他」のTBT濃度は，別に測定した筋肉中TBT濃度も合わせて補正して示した。

TBTの分析方法

飼育水中のTBTClはn-ヘキサンを用いて抽出し，n-プロピルマグネシウムブロミドを用いてプロピル誘導体に変換した後，キャピラリーカラム（Ultra-1）およびFPD検出器（Snフィルター使用）を装備したガスクロマトグラフ（GC-FPD）を用いて測定した。この方法の定量下限値は10ng/Lであり，添加回収率は96.7±1.2%（平均±標準偏差，n=3），それらの変動係数は1.2%

であった。

マミチョグ組織中TBTClの分析は高見ら（1987）の方法に準拠して測定した。すなわち，マミチョグ組織中のTBTClは1N塩酸メタノール・酢酸エチル（1：1，V/V）混液を用いて抽出した。抽出物を陰イオンと陽イオン交換樹脂を用いて精製した後に，飼育水中TBTClの分析と同様にプロピル誘導体に変換し，GC-FPD（Snフィルター使用）で測定した。この方法の定量下限値は0.005μg/gであり，また，添加回収率は97.9±1.3%（平均±標準偏差，n=3），それらの変動係数は1.3%であった。なお，濃度はTBT^{+}として湿重量当たりに換算して表示した。

成熟開始時期から産卵期における曝露試験

試験魚

前年の春に産卵・ふ化した後に瀬戸内海区水産研究所の屋内水槽で自然条件下で飼育した当歳魚を試験に用いた。試験は平成13年2月5日～5月7日の91日間（77日間の暴露試験とそれに続く14日間の排泄試験）実施した。すなわち，マミチョグの成熟開始時期から成熟，産卵期について試験を実施した。試験開始時の雌マミチョグの体長および体重は，それぞれ6.4±0.2cmおよび6.5±1.2gであり，雄マミチョグの体長および体重は，それぞれ6.5±0.3cmおよび7.2±0.9gであった。

暴露試験

雌対照区，雄対照区，雌暴露区および雄暴露区の4試験区を設定し，瀬戸内海区水産研究所に設置している流水式曝露試験装置を用いて暴露試験を行った。

試験水槽として角型ガラス水槽（容積約50L）を用い，雌対照区および雄対照区には，雌および雄それぞれ40尾を，また，雌曝露区および雄曝露区には55尾の雌あるいは雄マミチョグを収容した。砂および活性炭でろ過した自然海水を27L/hの流量で試験水槽に注水し，水槽内飼育水を1日に約13回換水させた。

上で述べた不応期における暴露試験と同様にTBTClを溶解させた中間薬液を，微量定量ポンプを用いて定量的に試験水に添加し，飼育水中のTBT濃度を一定に維持した。平成13年2月5日～4月23日の77日間の暴露試験を実施した。暴露試験終了後TBTClの添加を中止するとともに，TBTClを添加しない飼育水中で4月23日～5月7日まで14日間マミチョグを飼育し，TBTClの排泄を調べた。

暴露および排泄試験の間，試験魚を自然水温（91日間の試験の間に10.0℃から15.9℃に上昇した）および14時間明，10時間暗の明暗周期の条件下で飼育した。また，体重の2%の配合飼料（協和発酵(株)製初期飼料C-2000）を1日2回（朝と夕方）に分けて投与するとともに，毎朝残餌および糞を取り除いた。

対照区の飼育水は7日目，28日目，63日目および77日目に採取しTBT濃度を測定した。暴露区の飼育水中TBT濃度は，暴露試験期間中21および56日目を除いて毎週測定した。

対照区のマミチョグは経時的に3尾づつ取り上げ，解剖した後に肝臓，生殖腺，消化管および「その他」に分け，それぞれの部位をひとまとめにした。モノブチルスズ（MBT），ジブチルスズ（DBT）およびTBTのブチルスズ化合物（BTs）濃度は，91日目（排泄14日目）の試料について測定した。

暴露区のマミチョグは，経時的に3～5尾取り上げ，BTs濃度は，試験開始時，暴露試験の15日目，77日目および排泄試験の14日目（試験開始から91日）に取り上げたものを用いた。対照区と同様に肝臓，生殖腺，消化管および「その他」に分けて，取り上げたマミチョグをひとまとめにして分析用試料とした。

GSIの測定

魚体を解剖して分析用試料を調製する際に体重および生殖腺重量を測定し，次式により生殖腺指数（GSI）を計算した。

$$GSI\ (\%) = (\text{生殖腺重量}/\text{魚体重}) \times 100$$

飼育水TBTおよびマミチョグ中MBT，DBTおよびTBTの分析方法

飼育水中のTBT濃度は不応期における暴露試験において述べた方法に準拠して測定した。ただし，飼育水中TBT濃度が不応期の暴露試験に比較して高いため分析操作上定量限界値を20ng/Lとした。一方，不応期の暴露試験で採用した分析方法では，マミチョグ組織中のMBT，DBTおよびTBTを同時に分析することができないため，TBTおよび代謝物質は張野，福島（1994）が開発した方法に準拠して分析した。すなわち，マミチョグ組織中のBTsは1%トロポロン・アセトン溶液を用いて抽出し，抽出物をベンゼンに転溶させた後に，MBT，DBTおよびTBTのプロピル誘導体に変換後，フロリジルカートリッジカラムで精製した。濃縮した後にFPD検出器（Snフィルター使用）付きガスクロマトグラフ（GC）を用いて測定した。GCの分析条件は張野，福島（1994）に準拠し，その濃度はMBT^{3+}，DBT^{2+}およびTBT^{+}として湿重量当たりに換算して示した。この方法による定量下限値は，GC用試験溶液量の違いから3物質ともに対照区で0.02μg/g，暴露区で0.05μg/gであり，また，MBT，DBTおよびTBTの添加回収率は，それぞれ，99.0±4.9%，83.7±4.2%および94.8±3.7%（平均±標準偏差，n=5），変動係数はMBTで4.9%，DBTで5.0%，また，TBTで3.9%であった。

結果および考察

不応期における生物濃縮

飼育水中のTBT濃度

試験に用いた海水のTBT濃度は定量下限（10ng/L）未満であった。試験期間における飼育水中TBT濃度はTable1に示したように，雌暴露区で74～130ng/L，また，雄暴露区で85～130ng/Lの範囲で変動した。飼育水中TBT濃度の平均値±標準偏差は，雌暴露区で120±21ng/L，雄暴露区で110±19ng/Lであった。

Table 1. TBT concentration (ng/L) in rearing seawater of refractory period experiment

Exposure period(day)	Exposure experiment	
	Female	Male
initial	74	85
7	130	85
14	120	120
21	120	120
28	89	98
35	130	120
42	120	120
49	120	130
56	89	98
Mean±SD	120±21	110±19

魚体中TBT濃度の経時的変化と生物濃縮係数

曝露14～56日目の全魚体中TBT濃度をTable 2に示した。なお，56日目の濃度は，各部位のTBT濃度と各部位の重量比から算出した。雌暴露区の14日目，28日目，42日目および56日目の魚体中TBT濃度は，それぞれ，0.31μg/g，0.51μg/g，0.71μg/gおよび1.1μg/gであり，14～28日目および28～42日目において有意に上昇し（t検定，2～4週目$p<0.01$，4～6週目$p<0.05$），42～56日目においては有意差は認められなかった。また，雄曝露区も同様にTBT濃度は，14～28日目および28～42日目において有意に上昇し（t検定，$p<0.01$），42～56日目においては有意差は認められなかった。したがって，マミチョグ体内に取り込まれたTBTは42日目以降ほぼ平衡に達したと考えられた。試験開始前のマミチョグの魚体中TBT濃度は定量下限（0.005μg/g）未満であり，56日目の暴露区マミチョグの濃度を用いてTBTClの生物濃縮係数（BCF）を計算した。BCFは，Table 3に示したように，雌で9,200±1,700，また雄で9,100±640であり（平均±標準偏差，n=3），雌雄のマミチョグで有意に異ならなかった。TBTClの海産魚によるBCFはマダイで6,800±900であることが報告されており（山田，1992），マミチョグはマダイと同様にTBTClを高濃度に濃縮することが明らかであった。

Table 2. TBT concentration in whole body of mummichog exposed to waterborn TBTCl

Exposure period(day)	TBT concentration (μg/g wet wt)	
	Female	Male
14	0.31±0.03*2	0.36±0.03
28	0.51±0.03	0.57±0.05
42	0.71±0.10	0.90±0.09
56*1	1.1 ±0.20	1.0 ±0.07

*1 TBT concentrations were calculated by using concentration in each tissues and the percentage of each tissues to whole body
*2 Mean±SD (n=3)

TBT濃度および生物濃縮係数の組織・器官による差異

暴露56日目のマミチョグ各部位別のTBT濃度はTable 3に示したように，血液で特に高く雌雄のマミチョグでそれぞれ1.9μg/gおよび1.6μg/gであった。雌マミチョクでは肝臓，「その他」および生殖腺のTBT濃度は，それぞれ，1.2μg/g，1.1μg/gおよび1.0μg/gであり，血液に次いで高いが，これらの部位の濃度差は明確ではなかった。雌マミチョグの消化管TBT濃度は0.68μg/gと他の部位に比較して低かった。雄マミチョグについても，血液以外の部位別TBT濃度は，肝臓，「その他」，生殖腺および消化管，それぞれ，0.92μg/g，1.0μg/g，0.99μg/gおよび0.70μg/gであり，雌マミチョグに比較すると，消化管では濃度が低く，また，肝臓，生殖腺および「その他」におけるTBT濃度差は小さく，部位別TBT濃度は雌雄のマミチョグで類似する傾向であった。「その他」から筋肉を一部採取し，筋肉中濃度を測定した結果は，雌および雄それぞれ0.78μg/gおよび0.96μg/gであった。雌マミチョグの筋肉中TBT濃度は消化管と同等であり，雄マミチョグのそれは肝臓，生殖腺と同等であった。

部位別TBT濃度の有意差を一元配置分散分析をした後に多重比較を行って検定した。その結果，雌雄ともに血液中のTBT濃度は他の部位に比べ有意に高かった（Tukey，$p<0.01$）。本研究の結果は，酸化トリブチルスズ（TBTO）に暴露したマダイの組織・器官のTBT濃度が筋肉に比較して肝臓，鰓および表皮で高い傾向が認められるもののその差は小さいこと（Yamada and Takayanagi, 1992）およびマコガレイやヒラメの血清中にTBTが高濃度に検出される既往の報告（Oshima *et al.*, 1997; 大嶋ら，1998a; Oshima *et al.*, 1998b）に一致するものである。

TBTClの各部位におけるBCFをTable 3に併せて示した。雌マミチョグの各部位におけるBCFは，血液（全血），肝臓，「その他」，生殖腺，筋肉および消化管で，それぞれ15,800，10,000，9,200，8,300，6,500およ

Table 3. TBT concentration and BCF in several tissues and organs of mummichog exposed to waterborn TBTCl for 56 days

Tissue and organ	TBT Concentration(μg/g wet wt)[*4]		BCF	
	Female	Male	Female	Male
Blood	1.9±0.34[*5]	1.6±0.33	15800±2800	14500±3000
Liver	1.2±0.28	0.92±0.04	10000±2300	8400± 360
Residue[*1]	1.1±0.22	1.0±0.07	9200±1800	9100± 640
Gonad	1.0±0.13	0.99±0.11	8300±1100	9000±1000
Muscle	0.78±0.09	0.96±0.10	6500± 750	8700± 910
Digestive tract[*2]	0.68±0.16	0.70±0.05	5700±1300	6400± 450
Whole body[*3]	1.1±0.20	1.0±0.07	9200±1700	9100± 640

[*1] including muscle, gill, heart, kidney, bone, skin and airbladder
[*2] including gallbladder, spleen and adipose tissue aroud the digestive tract
[*3] TBT concentrations were calculated by using TBT concentration in each tissues and the percentage of each tissues to whole body
[*4] Three samples were analyzed
[*5] Mean±SD

び5,700であった。一方，雄マミチョグの各部位におけるBCFは，血液（全血），肝臓，「その他」，生殖腺，筋肉および消化管で，それぞれ，14,500，8,400，9,100，9,000，8,700および6,400であった。また，筋肉のBCFは，雌および雄それぞれ6,500および8,700であった。すなわち，雌雄のマミチョグの部位別のBCFは大差なく，不応期におけるTBTClのマミチョグへの生物濃縮は性によって著しく異なることはないと考えられた。

成熟開始時期から産卵期における暴露試験

飼育水中のTBT濃度

飼育水中のTBT濃度の経時変化をTable 4に示した。対照区の飼育水からはTBTは検出されず，TBT濃度は定量下限（20ng/L）未満であった。雌暴露区および雄暴露区の飼育水中のTBT濃度は，それぞれ，710～1,600ng/Lおよび630～2,500ng/Lであり，平均±標準偏差は雌暴露区で1,100±370ng/L，また，雄暴露区で1,400±670ng/Lであった。

生殖腺の成熟状況の変化

雄マミチョグのGSIはFig. 1に示すように対照区では実験開始時の4.1±1.6%から次第に大きくなり実験開始後63日後に8.5±2.6%の最大値を示した。その後，GSIは次第に小さくなり，実験終了時には5.8±2.3%に低下した。暴露区では，対照区と同様に変化し，実験開始時の5.0±0.4%から次第に大きくなり実験開始63日後には9.0±1.7%の最大値を示した。その後は対照区と同様に次第と小さくなり，実験終了時には5.9±0.6%に低下した。雄マミチョグは本研究の飼育条件では，実験開始後63日（4月9日）を中心として成熟の盛期に達しているとことが明らかであった。

実験終了時（排泄試験の14日目）に放精している雄マミチョグのGSIは，対照区で4.7±0.8%，暴露区で6.1±0.5%であり，実験終了時の未放精個体のGSIに比較すると小さかった。すなわち，最大成熟期を過ぎた雄マミチョグのGSIは放精により次第に低下することが示唆された。

Shimizu（1992）によると，三浦半島西側沿岸の荒崎において自然環境で飼育した雄マミチョグ当歳魚のGSIは2月から次第と大きくなり，精巣成熟のピークは4～5月でこの時のGSIは5～6.5%に達することが報告されている。精巣のGSI値およびその経時的変動傾向から暴露試験の後半から排泄試験期間中の雄マミチョグは充分成熟した状態であったと考えられる。

雄マミチョグのGSIおよびその経時的な変動傾向は対照区と暴露区で明確な相違は認められなかった。清水，木村（1992）はアゴハゼの成熟過程におけるTBTO

Table 4. TBT concentration (ng/L) in rearing seawater of maturating period experiment

Exposure period(day)	Control		Exposure experiment	
	Female	Male	Female	Male
1	<20	<20	1400	850
10			820	630
14			710	860
28	<20	<20	1500	2500
35			1600	2300
42			1600	2100
49			1300	1200
60	<20	<20	840	1100
70			830	940
77	<20	<20	870	1400
Mean±SD			1100±370	1400±670

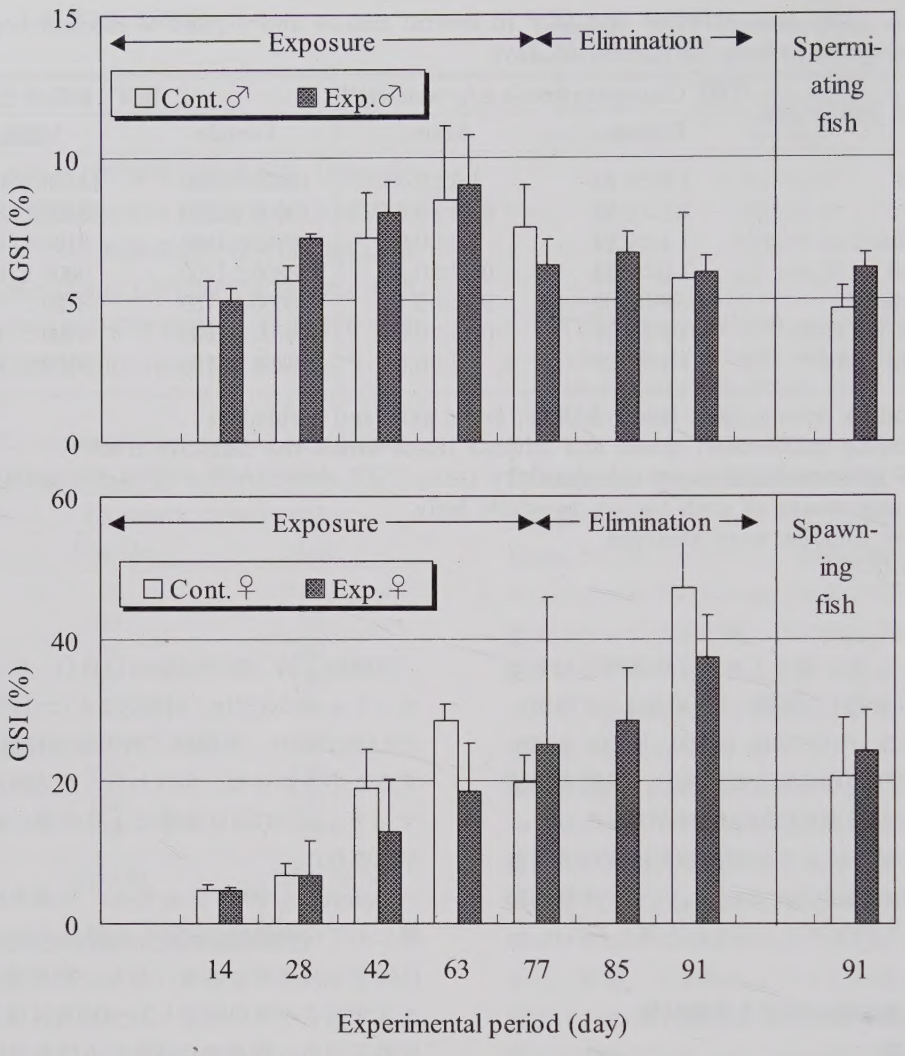

Fig. 1. Changes of gonad somatic index (GSI) during the experimental period

の影響を調べ，飼育水中TBT濃度が本研究（1,400±670ng/L）の1/10低い濃度（<100～200ng/L）において，雄アゴハゼのGSIが有意に低下した報告している。使用したTBT化合物が本研究（TBTCl）とアゴハゼの研究（TBTO）では異なるが，魚体内に取り込まれたTBTOとTBTClはTBTイオンとして存在すると推察すると，TBTOとTBTClの魚類精巣に対する作用機構は類似していると考えられる。したがって，精巣の成熟に対するTBT化合物の影響はマミチョグに比較してアゴハゼで強く発現することが示唆される。

雌マミチョグのGSIはFig. 1に示すように対照区では実験開始時の4.6±0.5%から次第に大きくなり実験終了時（5月7日）に47.1±7.2%の最大値を示した。一方，暴露区のマミチョグのGSIは対照区と同様な変化を示し，実験の終了時に最大のGSI（37.5±5.9%）に達した。実験の終了時には対照区および暴露区とも産卵している個体が認められ，本研究の飼育条件下では4月下旬から5月上旬が産卵期と考えられる。実験終了時（排泄試験の14日目）に産卵している雌マミチョグのGSIは，対照区で20.7±8.3%，暴露区で24.4±5.6%であり，GSIは産卵に伴って小さくなることが明らかであった。

上述したShimizu（1997）の研究によると，当歳のマミチョグの卵巣は3月から急激な発達を開始し，4月から6月に成熟がピークに達し，この時のGSIは16%～22%であることが報告されている。91日間の試験終了時の対照区および暴露区のGSI（それぞれ47.1%および37.5%）はShimizuが観察した値より大きいが，GSIの経時的変動から本研究に用いた雌マミチョグは，雄マミチョグと同様に暴露試験の後半から排泄試験期間において充分成熟した状態であったことが明らかである。

卵巣のGSIおよびその経時的な変動傾向は，雄マミチョグと同様に対照区と暴露区で明確な相違は認められなかった。TBTOのアゴハゼの成熟過程に対する影響を調べた清水，木村（1992）の研究では，雌アゴハゼのGSIの有意な低下は，飼育水中TBT濃度が本研究（1,400±670ng/L）よりもわずかに高い濃度（2,100±800ng/L）で認められたと報告されている。すなわち，雌マミチョグの卵巣の成熟に対するTBT化合物の影響はアゴハゼと大差ない傾向であった。TBT化合物の精巣および卵巣の成熟過程に対する影響は，アゴハゼとマミチョグの2魚種により異なることが示唆されるが，その原因については試験に用いたTBT化合物の種類と影響発現の関係などさらに詳細な検討が必要であると考えられる。

マミチョグ各組織中BTs濃度

マミチョグの各組織中のBTs濃度をTable 5および6に示す。Table 5に示したように，対照区の雌雄のマミチョグからはTBT濃度は定量下限（0.02 μg/g）未満であった。DBTは雌の肝臓並びの雄の精巣および消化管から検出され，その濃度はいずれも0.03 μg/gであった。これら以外の組織中DBT濃度は定量下限（0.02 μg/g）未満であった。MBTは雄の消化管から0.05 μg/gの濃度で検出されたが，これ以外の組織器官では検出されず，定量下限（0.02 μg/g）未満であった。

Table 6に示したように，TBTClに暴露した雌マミチョグからBTsが検出され，肝臓，消化管および「その他」のTBT濃度は暴露試験の15日目より暴露期間が長い77日目の方が高く，暴露期間の延長に伴って濃度が高くなり蓄積していることが明確であった。また，2週間の排泄試験の間に濃度が低下するために魚体から排泄されることが明らかであった。一方，卵巣中のTBT濃度は他の部位とは異なる変化を示した。すなわち，排泄実験においてもその濃度は低下せず，排泄試験終了時においても77日間の暴露試験終了時と同じ濃度を維持していた。すなわち，卵巣のGSIは91日間の試験期間の終了時まで次第に大きくなったために体内に取り込まれたTBTClの一部分は，排泄試験期間中に卵巣へ移行したことが推察されるが，魚体内での移行および再分布は今後追求する必要のある重要な課題と考えられる。

TBTClに暴露した雄マミチョグの肝臓，消化管，精巣および「その他」のTBT濃度は暴露15日目より77日目の方が高く，暴露試験期間中に濃縮・蓄積された。77日間の暴露試験終了時における，肝臓，精巣，消化管および「その他」のTBT濃度は，それぞれ，1.8 μg/g，2.1 μg/g，1.6 μg/gおよび1.0 μg/gであり，精巣で高い傾向が認められた。一方，精巣中TBT濃度2.1 μg/gは，卵巣中濃度に比較して著しく高かった。精巣中TBT濃度は排泄試験期間中に2.1 μg/gから1.1 μg/gへと半減したが，排泄試験期間中の肝臓，消化管およびその他におけるTBT濃度の変化は小さかった。特に，消化管および「その他」のTBT濃度はわずかに増加傾向であり，排泄試験期間中に低下しなかった。この濃度変化の傾向は雌マミチョグとは異なるが，その原因については明らかでない。

精巣のGSIは生物濃縮試験63日で最大値，9.0±1.7%を示し，その後低下している。したがって，排泄試験期間中には精巣は次第に退行しており，このために精巣中TBT濃度は卵巣とは異なり排泄試験期間中2.1 μg/gから1.1 μg/gへ次第に低下したと考えられる。

MBT，DBTおよびTBTの合計であるΣBTsの雌マミチョグ肝臓中濃度は，15日で0.49 μg/g，77日で3.18 μg/g，91日で4.90 μg/gであり，排泄試験期間においてもその濃度は増大した。一方，ΣBTsに対するTBT代謝物質（MBTおよびDBTの合計）の割合は試験期間15日の18.4%から91日には88.8%に増大した。雄マミチョグ肝臓におけるΣBTs濃度およびΣBTsに対するTBT代謝物質の割合の経時変化の傾向は雌マミチョグと同様であった。また，マミチョグで認められた結果は，TBTOに暴露されたマダイ肝臓において報告されたMBT，DBTおよびTBT濃度の経時変化（山田，1999）と同様であった。これらの結果から，マミチョグにおいても魚体内に蓄積されたTBTClは他の海産魚

Table 5. Concentrations of butyltin compounds in several tissues and organs of the control mummichog(μg/g wet wt)

Tissue and organ	Concentration in female			Concentration in male		
	MBT	DBT	TBT	MBT	DBT	TBT
Liver	<0.02	0.03	<0.02	<0.02	<0.02	<0.02
Gonad	<0.02	<0.02	<0.02	<0.02	0.03	<0.02
Digestive tract	<0.02	<0.02	<0.02	0.05	0.03	<0.02
Residue	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02

類と同様に主として肝臓において代謝されることが示唆された。

ΣBTsに対するTBT代謝物質の割合は，卵巣で25%，精巣で9.8～17.1%，雌雄の消化管で24.5～47.6%，雌雄の「その他」で0～38.8%であり，その割合は消化管で大きい傾向が認められる。肝臓で生成されたTBT代謝物質は，胆汁とともに消化管に分泌されるために消化管ではΣBTsに対してTBT代謝物質の占める割合が大きくなると考えられる。

不応期と成熟期における体内分布の比較

不応期に行った試験による雄および雌マミチョグの肝臓，生殖腺，消化管および「その他」におけるBCFは，それぞれ，8,400～10,000，8,300～9,000，5,700～6,400および9,100～9,200であった（Table 3）。一方，成熟期に行った試験によるこれらの値は780～1,300，220～1,500，1,100～1,300および700～2,200であり，不応期のBCFは成熟期の試験で得た値に比較して約4～40倍大きかった。これらの2回の試験は，マミチョグの大きさ，試験水温および飼育水中のTBT濃度等が異なる試験条件下で実施されており，これらの試験条件の差異はBCFを変化させる要因となると考えられる。特に，飼育水中濃度はBCFを変動させる主要な要因であり，その濃度が高いほどBCFが小さくなることが報告されている（山田，1999）。本研究では，成熟期における試験の飼育水中TBT濃度（1,100～1,400ng/L）は不応期の試験（110～120ng/L）に比べて約10倍高いために，成熟期マミチョグの組織・器官におけるTBTClのBCFは，不応期の試験に比較して小さくなったと考えられ，不応期と成熟期におけるBCFの差異は成熟に伴ったマミチョグの生理的変化に起因するものではないと推察される。

本研究で対象とした組織・器官におけるBCFは，不応期の試験では雌雄により大差なかった。一方，成熟期の試験では，消化管におけるBCFは雌雄による差が認められないが，肝臓および生殖腺におけるBCFは雄マミチョグにおいて，また，「その他」におけるBCFは雌マミチョグにおいて大きかった。特に，成熟期マミチョグの精巣にはTBTClが高濃度に蓄積され，BCFが卵巣に比較して7倍大きくなることが明らかであった。

TBTClを含有する飼育水で魚類を飼育すると，飼育水中TBTClは主として鰓を経由して吸収され，血液を通して魚体全体に移行する。肝臓における薬物代謝酵素の作用によりその一部分は代謝産物（TBT化合物の場合はMBTおよびDBT）に変換され，TBTClおよび代謝産物は血液を介して再循環する。TBTClは魚体内ではTBT$^+$として存在すると考えられており，また，血液中ではある種のタンパク質に結合した状態で溶存することが確認されている（大嶋ら，1998a; Oshima *et al.*, 1998b; Shimasaki *et al.*, 2002）。一方，生殖腺の成熟過程において精巣や卵巣において生殖腺の成熟に関与する特異な物質（タンパク質等）が合成されることが報告されている（Mochida *et al.*, 2002a; Mochida *et al.*, 2002b; Miura *et al.*, 2002; Andreassen *et al.*, 2002）。これらの生成された物質が，血液中のTBT－タンパク質複合体あるいはTBTに対して強い親和性を有すると，血液中TBTの精巣への

Table 6. The concentrations of butyltin compounds and BCF in several tissues and organs of mummichog exposed to waterborn TBTCl

Tissue and organ	Experimental period(day)		Concentration in female(μg/g wet wt) MBT	DBT	TBT	ΣBTs	BCF of TBT	Concentration in male(μg/g wet wt) MBT	DBT	TBT	ΣBTs	BCF of TBT
Liver	Exposure	15	<0.02[*1]	0.09	0.40	0.49		0.11	<0.05	0.31	0.31	
		77	0.02	2.0	0.86	3.18	780	0.39	3.3	1.8	5.49	1300
	Elimination	91	0.65	3.7	0.55	4.90		0.52	4.4	1.3	6.22	
Gonad	Exposure	15	<0.05	<0.05	0.38	0.38		0.06	<0.05	0.29	0.29	
		77	<0.05	<0.05	0.24	0.24	220	0.12	0.23	2.1	2.45	1500
	Elimination	91	<0.05	0.08	0.24	0.32		<0.05	0.12	1.1	1.22	
Digestive tract	Exposure	15	0.07	<0.05	0.27	0.34		<0.05	<0.05	0.75	0.75	
		77	0.27	0.28	1.4	1.95	1300	0.15	0.37	1.6	2.12	1100
	Elimination	91	0.18	0.21	0.43	0.82		0.19	0.38	1.7	2.27	
Residue	Exposure	15	<0.05	<0.05	0.58	0.58		0.14	<0.05	0.22	0.36	
		77	0.06	0.23	2.4	2.69	2200	<0.05	0.15	1.0	1.15	700
	Elimination	91	<0.05	0.11	0.67	0.78		<0.05	0.16	1.7	1.86	

[*1] The concentration below the detection limit was treated as zero in the calculation of ΣBTs

移行および高濃度蓄積が引き起こされると考えられる。今後，精巣の成熟過程で生成される物質とTBTの親和性および精巣におけるTBT化合物の存在状態等を詳細に検討することが，TBT化合物の精巣への蓄積および精巣の発達阻害の解明において重要である。

TBT化合物による精巣の発達阻害は卵巣に障害を引き起こす濃度に比較すると1/100の低濃度で引き起こされることが確認され（清水，木村，1992），TBT化合物の魚類生殖過程への影響は精巣に対して特に顕著であることが報告されている。飼育水中のTBT化合物が成熟期には精巣に高濃度に蓄積される本研究結果は，TBT化合物が精巣に対して特に著しい影響を及ぼすことを傍証する情報であるが，TBT化合物の精子形成等雄魚の生殖過程に対する影響と作用機構を詳細に検討することが今後の重要な課題であると考えられる。

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成熟期マミチョグの長期飼育試験による4-*tert*-オクチルフェノールの生物濃縮と体内分布

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Bioconcentration and distribution of 4-*tert*-octylphenol in mummichogs (*Fundulus heteroclitus*) exposed during the maturation period

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Abstract Male and female mummichogs (*Fundulus heteroclitus*) were reared for 56 days in order to understand possible sexual differences in intake, elimination and tissue distribution of 4-*tert*-octylphenol (OP). The bioconcentration factors (BCF) for the entire body of the females and the males were 34±11 and 23±5.1, respectively. The difference was negligible. These BCFs were one order smaller than those reported previously for carp and Japanese medaka. The OP concentration was the highest in the digestive tract and the lowest in the blood of both the males and females. The BCF in the studied tissue and organs of the male fish was similar to that of the female fish. These results suggest that the bioconcentration of OP is independent of sex. OP was excluded rapidly from the fishes' tissues and organs in the elimination experiment, with the exception of the ovaries. The concentration of 0.08 μg/g wet weight in the ovaries was observed on the 14th day of the elimination experiment. OP in the ovaries is retained for a long period compared to other fish tissues and organs. These results suggest that OP can inhibit developmental processes in the early life stage of the next generation of fish.

Key Words: mummichog, maturation, bioconcentration, tissue distribution, 4-*tert*-octylphenol

アルキルフェノールは，脂溶性フェノール樹脂や非イオン界面活性剤（アルキルフェノールポリエトキシレート，APE）の構成物質あるいは有機合成中間体として化学工業において広く使用されている（経済産業省，2002）。平成10年度の4-*tert*-オクチルフェノール（OP）の製造量は，7,863トンと報告されており，多量に製造・使用されている化学物質である（経済産業省，2002）。

OPは，下水処理過程においてAPEの分解により生成されることが明らかにされた（Ahel *et al.*, 1994a, 1994b）。一方，東京周辺および名古屋市の河川水中からもAPEの分解中間物質とともにアルキルフェノールが検出されている（磯部ら，1999）。平成10年度に環境省が実施した調査結果（環境省，2000）を用いて，OPの水質，底質および魚類からの検出率と検出濃度を整理すると（山田，藤井，2001），水質からは，405試料のうち228試料から検出され，その濃度が＜0.01～13 μg/Lであった。また，底質での検出率および濃度は，それぞれ7％および＜0.005～0.038 μg/g，同じく魚類

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でそれぞれ11%および＜0.025～0.18 μg/gであった。環境省の調査からも明らかなようにOPはしばしば環境水から検出される物質であり，小島，渡辺（1998）の研究によると，名古屋市内河川から＜0.1～0.41 μg/Lの濃度で検出されている。OPの海水中濃度は＜0.01～0.06 μg/Lであり，調査した範囲での最大濃度，0.06 μg/Lは東京湾および大阪湾で観測された（環境省，2000）。調査データは乏しいが，海水中濃度は都市河川水中濃度よりは低く，OPが陸上から流入することが示唆された。

酵母を用いたツーハイブリッド試験法による研究により，本物質がエストロゲン様作用を有することが報告され（Routledge and Sumpter, 1996），ゼノエストロゲンの一つとして魚類の雌化など魚類生殖機能に対する影響実態および作用機構に関して多くの研究が進められてきた。これらの研究により，本物質が卵黄タンパク質の前駆物質であるビテロジェニンを誘導するとともに，雄ニジマスの精巣の発達阻害を引き起こ越すことが明らかにされた（Jobling *et al.*, 1996）。さらに，ふ化直後のヒメダカ仔魚に対する本物質の暴露が精巣卵を惹起し，性分化期における暴露が精巣卵を特に顕著に引き起こすことが報告され（Gray *et al.*, 1999a），OPのゼノエストロゲンとして作用がin vivoでも確認された。OPを暴露したヒメダカから産卵された卵を用いて受精率やふ化率等の次世代仔魚の発生に対する影響を調べた研究（Gray *et al.*, 1999b）では，OPが受精率およびふ化率を低下させるとともに，発生過程に各種の影響を及ぼし，循環系，眼および鰾の欠損などの奇形を引き起こすことが明らかにされた。すなわち，OPは次世代の仔魚の発生に対しても影響を及ぼすことが指摘された。

次世代の仔魚の発生に対する影響を明らかにするためには，魚卵を中心として生殖腺におけるOPの生物濃縮を明らかにすることは重要な課題である。しかし，本物質のlogKowが1.4（経済産業省，2002）と小さいことから生物濃縮係数（BCF）も小さいと考えられるために，魚類におけるOPの生物濃縮はほとんど検討されていない。わずかに，BCFはコイ（通商産業省，1992）およびヒメダカ（Tsuda *et al.*, 2001）を用いて研究され，BCFはコイで135～469，また，ヒメダカで261±62（平均±標準偏差）と報告されている。本物質が海域からも検出されるにも拘わらず，海産魚による生物濃縮の実態はほとんど研究されていない。

そこで，本研究では北アメリカ大陸東岸汽水域に生息する広塩性小型魚であるマミチョグ（*Fundulus heteroclitus*）を試験魚として用いて長期の飼育実験を行い，海産魚についてのOPのBCFおよび蓄積部位を明らかにするとともに，各部位における蓄積，排泄の動態を把握してOPの魚類生殖機能への影響解明のための基礎的情報を得ることを目的とした。

なお，本研究で試験魚として用いたマミチョグは，アメリカ公衆衛生協会が定めたStandard Methods（1998）において魚類繁殖阻害試験の推奨魚として採用されており，化学物質の魚類に対する有害性解明の研究（Dube and MacLatchy, 2001など）に用いられている。我が国においてマミチョグの屋外飼育環境下における成熟産卵周期はShimizu（1997）により研究され，GSIの季節変化および繁殖時期が解明されている。したがって，欧米各国において実施された研究の結果と比較検討することができ，さらに，成熟開始時期からの暴露試験計画の立案が容易なために試験魚としてマミチョグを選定した。

材料と方法

4-*tert*-オクチルフェノール（OP）

Aldrich 社製の試薬（純度97%）を購入し，暴露試験に用いた。暴露試験開始までは冷暗所に保存した。一方，和光純薬工業㈱製の標準品（純度97.6%）を化学分析用標準物質として使用した。

試験魚

平成12年春に産卵・ふ化した後，瀬戸内海区水産研究所の屋内水槽で自然条件下で飼育した2年魚を試験に用いた。試験は平成14年3月19日～5月14日のマミチョグの成熟・産卵期に56日間（42日間の暴露濃縮試験とそれに続く14日間の排泄試験）実施した。試験開始時の雌マミチョグの体長および体重は，それぞれ，8.1±0.4cmおよび17.1±1.7gであり，また，雄マミチョグの体長は7.5±0.2cm，体重は14.7±0.3gであった。

成熟・産卵期におけるOP曝露試験

雌対照区，雄対照区，雌暴露区および雄暴露区の4試験区を設定し，瀬戸内海区水産研究所に設置している流水式暴露試験装置を用いてOPの暴露試験を行った。

試験水槽として角型ガラス水槽（容積約50L）を用い，雌雄の対照区には，雌あるいは雄マミチョグをそれぞれ33尾，同様に，暴露区には雌あるいは雄マミチョグをそれぞれ51尾収容した。砂および活性炭でろ過した自然海水を22～23L/hの流量で試験水槽に注水し，水槽内飼育水を1日に約11回換水させた。

OPのアセトン溶液の一定量を撹拌・混合させながら水道水に溶解させた中間薬液を微量定量ポンプを用いて定量的に飼育試験水に添加し，飼育水中のOP濃度を一定に維持した。平成14年3月19日～4月30日に42日間の暴露試験を行い，OPの生物濃縮を調べた。暴露試

験終了後OPの添加を中止し，清浄な飼育水中で5月14日まで14日間マミチョグを飼育し，OPの排泄を調べた。

暴露および排泄試験の間，試験魚を自然水温（試験期間中に12.2℃から17.6℃に上昇した）および14時間明，10時間暗の明暗周期の条件下で飼育した。また，体重の2％の配合飼料（協和発酵(株)製初期飼料C-2000）を1日2回（朝と夕方）に分けて投与するとともに，毎朝残餌および糞を取り除いた。

対照区の飼育水は試験の10日目および35日目に採水し，試験用水中のOPを測定した。暴露区の飼育水中OP濃度は暴露試験の3日目，10日目，28日目および35日目に測定した。

対照区のマミチョグは経時的に3尾づつ取り上げ，採血した後に解剖して肝臓，生殖腺，消化管およびその他の部位（以後「その他」と言う）に分け，3尾のマミチョグのそれぞれの部位を1試料としてひとまとめにし，分析用試料とした。対照区の魚体中OP濃度は実験終了時の試料について測定した。

暴露区のマミチョグは経時的に3尾取り上げた。対照区と同様に血液，肝臓，生殖腺，消化管および「その他」に分けて，3尾のマミチョグのそれぞれの部位を1試料としてまとめて分析に供した。暴露試験の終了時の42日目には，マミチョグを3尾取り上げ，個体別に各部位別OP濃度を測定した。なお，暴露区の魚体中OP濃度は，7日目，28日目，42日目，49日目（排泄7日目）および56日目（排泄14日目）に測定した。

GSIの測定

魚体を解剖して分析用試料を調製する際に体重および生殖腺重量を測定し，次式により生殖腺指数（GSI）を計算した。

$$GSI\ (\%) = (\text{生殖腺重量}/\text{魚体重}) \times 100 \qquad (1)$$

OPの分析方法

飼育水

OP濃度は蛍光検出器付き高速液体クロマトグラフ（HPLC）を用いて測定した。HPLCの測定条件は，環境省（環境省，2001）に準拠し、予備試験の結果からTable 1のように定めた。飼育水中OP濃度は、設定濃度から機器の検出限界以上であると推察されるので，抽出，濃縮，精製をせず，海水試料を直接HPLCに注入して測定した。

Table 1. Analytical condition of HPLC

Apparatus	Waters 2690 alliance
Column	Inertsil ODS-3 (ϕ4.6×150mm)
Mobil phase	Acetonitril/water (8:2)
Flow rate	1.0mL/min
Sample volume	20 μL
Detector	Waters 474
Wavelength	excitation, 235nm, emission, 295nm

マミチョグ

組織中OPはアセトニトリルを用いて抽出し，抽出物をアセトニトリル飽和n-ヘキサンで脱脂し，ロータリーエバポレーターで濃縮した。アセトニトリルで定容した後，HPLCを用いてOP濃度を測定し，湿重当たりの濃度に換算して示した。HPLCの測定条件は飼育水中OPの分析と同様であった。

なお，対照区の「その他」を用いて添加回収試験を行った結果，回収率は93.3±3.1％（平均±標準偏差、n=4）であり，それらの変動係数は3.3％であった。

結果および考察

飼育水中のOP濃度

飼育水中のOP濃度はTable 2に示すように，暴露試験期間において雌暴露区では60～120 μg/L，また，雄暴露区では67～180 μg/Lであった。平均±標準偏差は，雌曝露区および雄暴露区それぞれ87±25 μg/Lおよび130±47 μg/Lであり，雌暴露区および雄暴露区の変動係数は，それぞれ，28.7％および36.1％であった。飼育水中のOP濃度が大きく変動した原因は明確でないが，本物質が疎水性を示すとともに，吸着しやすい性質を有することも濃度変動の一因と考えられる。雌雄マミチョグの対照区の飼育水にはOPは検出されず，定量下限（5 μg/L）未満であった。

Table 2. OP concentration in the rearing seawater

Exposure period(day)	OP concentration(μg/L)			
	Control		Exposure experiment	
	Female	Male	Female	Male
3			60	67
10	<5	<5	88	140
28			79	120
35	<5	<5	120	180
Mean±SD			87±25	130±47

試験魚の成熟状況

対照区および暴露区の試験魚の成熟の程度をGSIを用いて調べ，試験期間におけるGSIの変化をFig. 1に示した。暴露雄魚のGSIは試験開始時の6.8％から次第に大きくなり，試験の21日目に8.1±3.2 ％，さらに35日目に8.8±0.9％と最大値を示し，その後時間の経過とともに次第に小さくなった。Shimizu（1992）によると，三浦半島西側沿岸の荒崎において自然環境下の屋外水槽で飼育した雄マミチョグ当歳魚のGSIは2月から次第に大きくなり，精巣成熟のピークは4～5月

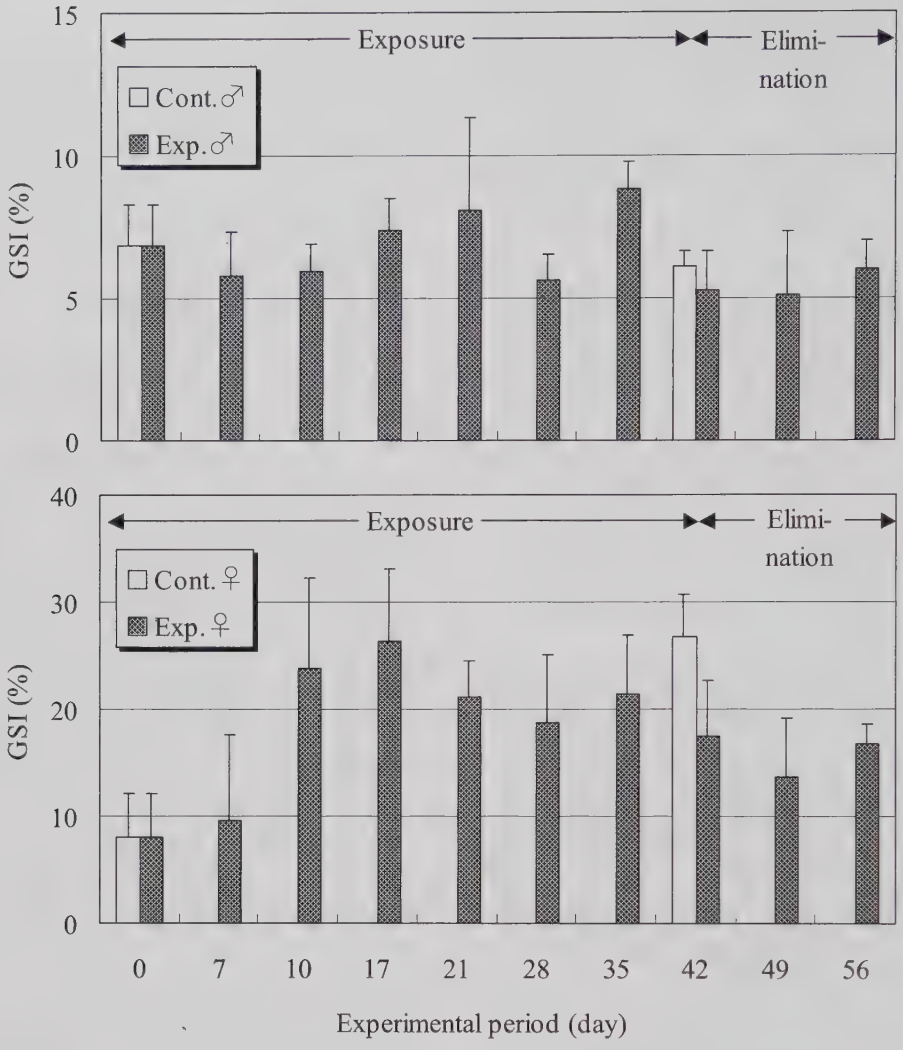

Fig. 1. Changes of gonad somatic index (GSI) during the experimental period

でこの時のGSIは5～6.5%に達することが報告されている。したがって，本研究に使用した雄マミチョグは暴露試験開始時にはすでに成熟していたと考えられる。

暴露試験終了時（42日目）の雄マミチョグのGSI（5.3±1.4%）は，対照区のそれ（6.1±0.5%）と大差ないために，精巣の発達はOPの暴露の影響を受けないことが明らかであった。

雌のGSIは，実験開始時（4月30日）の8.1%から，10日目（3月29日）の23.8%，17日目には26.3%と次第に大きくなり，17日目に最大値を示した。その後，21.4%から13.6%との間を変動した。Shimizu（1997）の研究によると，当歳のマミチョグの卵巣は3月から急激な発達を開始し，4月から6月に成熟がピークに達し，この時のGSIは16～22%であることが報告されている。これらの結果から，本研究に用いた雌マミチョグは暴露試験の10日目には充分成熟していたと考えられる。暴露試験42日目の対照区の雌マミチョグのGSI，26.7±4.1%は，暴露区のGSI，17.5±5.2%に比べて大きい傾向であった。しかし，35日目の暴露区の雌マミチョグのGSIは21.4±5.5%であり，42日目の対照区の雌マミチョグのGSIと大差ないために，本研究の濃度のOP暴露は雌の成熟を抑制しないことが示唆される。

OPのマミチョグによる生物濃縮

対照区マミチョグ各部位のOP濃度は，いずれの組織および器官についても定量下限（0.05 μg/g）未満であった。

暴露区マミチョグ各部位のOP濃度および全魚体に対する各部位の割合から計算した全魚体中OP濃度をTable 3に合わせて示した。OPに暴露した雌では，7日目，28日目および42日目における全魚体中OP濃度は，それぞれ，3.8，3.3および1.9±0.23 μg/gであった。一方，雄の全魚体中濃度は，7日目で2.9 μg/g，28日目

で2.4 μg/g，また，42日目で3.7±0.48 μg/gであった。全魚体および各部位におけるOP濃度は，雌マミチョグでは7日目と28日目では大差なく，同レベルであったが，42日目にはその濃度は低下した。しかし，雄マミチョグでは42日目に最も高いOP濃度を示した。

魚体内の化学物質の動態は，化学物質の物性、特に水・オクタノール分配係数（Kow）に依存し，Kowの小さい水溶解度の高い物質は排泄速度定数（k_2）が大きく，魚体から排泄されやすいことが経験的に知られている（Spacie and Hamelink, 1982）。logKowが1.4（経済産業省，2002）のOPは，k_2が大きいために，魚体中濃度は短時間で飼育水中濃度と平衡状態が達することが（2)式（Hawker and Connell, 1985）により明らかである。

$t_{eq}=4.605\times1/k_2$ あるいは　$\log t_{eq}=0.663-\log k_2$　(2)

Tsuda *et al.*（2001）の研究によると，ヒメダカで測定したOPのk_2は$0.09h^{-1}$と報告されており，魚体中濃度が飼育水中濃度と平衡に達し，一定になるまでに要する時間（t_{eq}）は，約2.1日間と推定される。すなわち，魚体中OP濃度は数日で飼育水中OP濃度と平衡状態に達するために，魚体中濃度は，飼育水中濃度の短期変動の影響を受けて変動することが考えられる。本研究の飼育水中OP濃度は雌雄の両実験区において35日目に最も高い濃度が測定されたが，35日から42日の間の飼育水中濃度は不明であり，この期間における濃度変化が42日目の魚体中OP濃度にも影響していることが推察される。

上述したようにOPの魚体中濃度は短時間の暴露試験の間に平衡状態に達すると考えられるので、暴露試験開始後７日目，28日目及び42日目の魚体中濃度はいずれも飼育水濃度に対して平衡状態に達した濃度と考えられる。したがって，飼育水中のOP濃度の短期変動に伴った魚体中濃度の変動を補正するために，７日目，28日目および42日目の魚体中濃度の平均値（以下，暴露期間平均魚体中OP濃度）を求めた。

７日目および28日目の魚体中濃度は３個体を合わせて調製した試料について測定したために，得られた濃度は３個体の平均値と考えられる。一方，42日目の魚体中濃度は個体毎に測定したために，暴露期間平均魚体中OP濃度は，まず42日目の魚体中平均濃度（42日平均濃度）を求め，さらに7日目および28日目の濃度並びに42日平均濃度の平均値を求める方法で計算した。

このようにして計算した暴露期間平均魚体中OP濃度と暴露試験期間中の飼育水中平均OP濃度（Table 2）の比から暴露試験期間中の平均的なBCF（以下平均BCF）を計算した。マミチョグ全魚体に対するOPのBCFは，暴露試験期間中に雌マミチョグで22～44，また，雄マミチョグで18～28の間を変動したが，雌雄のマミチョグの全魚体に対する平均BCFは，Table 3 に示したようにそれぞれ，34±11および23±5.1であった。平均BCFは雌雄のマミチョグで著しい差異が認められないために，OPのマミチョグ全魚体に対する生物濃縮は，マミチョグの性により著しく異ならないことが明らかであった。

マミチョグのBCFは，コイの135（飼育水中OP濃度が10 μg/L）および469（飼育水中OP濃度が100 μg/L）（経済産業省，2002）ならびにヒメダカの261（飼育水中濃度は4.7 μg/L）（Tsuda *et al.*, 2001）に比べて約１オーダー小さかった。コイやヒメダカに比較してマミチョグはOPを高濃度に蓄積しないことが示唆されるが，魚種による差異を解明するためには飼育水中のOP濃度や試験水温などの試験条件を同一にした状態での詳細な試験の実施が必要であると考えられる。

マミチョグ各部位へのOPの蓄積

雌マミチョグの各部位における暴露試験の全期間を通した平均OP濃度はTable 3 に示したように，消化管で17±7.6 μg/g，肝臓で3.0±1.8 μg/g，卵巣で2.3±0.4 μg/g，「その他」で1.9±0.36 μg/g，また，血液で0.71±0.25 μg/gであった。一方，雄マミチョグでは，平均OP濃度は，消化管で11±2.4 μg/g，肝臓で6.5±1.4 μg/g，「その他」で2.5±0.73 μg/g，精巣で2.1±0.66 μg/g，また，血液で0.97±0.24 μg/gであった。OP濃度は雌雄ともに消化管で特に高く，血液で最も低かった。消化管と血液以外の各部位におけるOP濃度は大差なかった。42日目の数値を用いて各位別OP濃度の有意差を検定すると，消化管の濃度が他の部位に比較して有意（Tukey, $p<0.01$）に高く，また，血液中濃度が他の部位に比較して低い傾向であることが明らかであった。

暴露試験期間を通した平均OP濃度と飼育水中OP濃度の平均値から求めた平均BCFをTable 3 に示すが，平均BCFは雌雄のマミチョグともに消化管で大きく，OPが消化管に高濃度に蓄積されることが明らかであった。OPは解離性基や加水分解を受けやすい化学結合を分子内に有しないために生体内では分子として存在するために，生物体内ではタンパク質等へ結合するよりは生物体内の脂質画分へ蓄積されると考えられる（経済産業省，2002)。本研究では，消化管周辺の腹腔内脂肪も消化管として分析したために，OPを高濃度に蓄積した腹腔内脂肪が消化管のOP濃度を高めた原因の一つと考えられる。

一方，海産魚は浸透圧調節のために飲水することが知られている。OPを含有する海水の飲水および消化管からのOPの吸収が，消化管のOP濃度を高める原因と

Table 3. Changes of OP concentration in sevral tissues and organs and average BCF

	Tissue and organ	OP concentration(μg/g wet wt) Exposure period (day) 7	28	42	Average concentration during exposure	Average BCF during exposure	OP concentration(μg/g wet wt) Exposure period (day) 7	14
♀	Blood	0.77	0.93	0.44±0.14[*2]	0.71±0.25	8.2±2.9	<0.05	<0.05
	Liver	4.6	3.2	1.1±0.62	3.0±1.8	34±21	0.11	<0.05
	Gonad	2.7	2.4	1.9±0.32	2.3±0.4	26±4.6	0.30	0.08
	Digestive tract	22	21	8.4±1.1	17±7.6	200±87	0.68	<0.05
	Residue	2.0	2.2	1.5±0.46	1.9±0.36	22±4.1	0.14	<0.05
	Whole body[*1]	3.8	3.3	1.9±0.23	3.0±0.98	34±11	0.21	<0.05
♂	Blood	0.99	0.72	1.2±0.22	0.97±0.24	7.5±1.8	<0.05	<0.05
	Liver	8.1	5.8	5.5±1.7	6.5±1.4	50±11	0.19	<0.05
	Gonad	2.0	1.5	2.8±1.1	2.1±0.66	16±5.1	<0.05	<0.05
	Digestive tract	9.6	10	14±0.58	11±2.4	85±18	0.20	<0.05
	Residue	2.2	1.9	3.3±0.31	2.5±0.73	19±5.6	<0.05	<0.05
	Whole body[*1]	2.9	2.4	3.7±0.48	3.0±0.66	23±5.1	<0.05	<0.05

[*1] OP concentrations were calculated by using OP concentration in each tissue and percentage of each tissue to whole body

[*2] Mean ±SD, n=3

も考えられる。酸化トリブチルスズ（Yamada and Takayanagi, 1992）および塩化トリブチルスズ（堀ら，2004）のマダイやマミチョグの組織・器官における蓄積濃度を検討した研究では，魚体内で各種のイオンあるいはタンパク質等に結合して存在し（Davies and Smith, 1980），脂質に対する親和性が低い（山田，1999）と考えられているトリブチルスズ化合物が消化管に特に高濃度に蓄積されなかったことが明らかにされている。すなわち，トリブチルスズ化合物を含有する飼育水を飲水しても消化管中トリブチルスズ濃度が消化管以外の部位における濃度よりも低いために，消化管中濃度に対する飲水の影響は小さいと推察される。したがって，生体内では分子として存在すると考えらるOPの消化管への高濃度蓄積に対して，マミチョグによる飼育水の飲水よりは消化管周辺腹腔内脂肪への蓄積が寄与していると考えられる。今後，腹腔内脂肪組織中のOP濃度の測定などさらに詳細な解析が必要である。

Table 3 に示した各部位における平均BCFを雌雄のマミチョグで比較すると，雌マミチョグでは消化管で，また，雄マミチョグでは肝臓で大きい傾向である。しかし，それらの標準偏差を考慮すると，肝臓および消化管における平均BCFは雌雄のマミチョグで有意に異なるとは考えられない。さらに，消化管および肝臓以外の部位および全魚体における平均BCFは雌雄のマミチョクで明確な差異は認められなかった。以上の結果から，OPの生物濃縮はマミチョグの性により著しく異なることはないと考えられた。

マミチョグからのOPの排泄

排泄試験期間中の魚体中OP濃度の経時変化をTable 3 に示した。排泄試験14日目には，卵巣を除けば雌雄のマミチョグからOPは検出されなかった。排泄試験 7 日目には，雄の血液，精巣および「その他」並びに雌の血液中OP濃度は定量限界（0.05μg/g）未満であり，雄の方が排泄速度が大きい傾向であった。

排泄試験 7 日目の雌の消化管，卵巣，「その他」および肝臓中OP濃度は，それぞれ，0.68μg/g，0.30μg/g，0.14μg/gおよび0.11μg/gであり，暴露試験期間中平均OP濃度の 4 %が消化管に，13%が卵巣に，7.4%が「その他」に，また，3.7%が肝臓に残留していた。卵巣には排泄試験14日目においても0.08μg/gのOPが検出され，暴露試験期間中平均OP濃度の3.5%が残留していた。雄では、排泄試験 7 日目の消化管と肝臓中OP濃度が0.20μg/gおよび0.19μg/gであり，暴露試験期間中平均OP濃度のそれぞれ1.8%および2.9%が残留していた。

以上の結果から，自然環境において実際に観察される濃度よりも10倍以上高い濃度での暴露試験ではあるが，消化管および卵巣に蓄積されたOPは排泄され難く，特に，卵巣に蓄積されたOPは精巣に比較して長期間魚体内に残留することが明らかであり，次世代仔魚の初期発生への影響が危惧される。今後，卵中のOP濃度と次世代仔魚の発生異常との関係などをさらに追求し，OPの世代を越えた影響をさらに検討する必要がある。

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技術報告

水産生物遺伝資源保存事業海洋微生物部門ベースコレクションの生物性状[*1]

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Characterization of the base collection strains preserved in the Marine Microorganism Division, a sub-bank of the Microbial Culture Collections, Fish Research Agency.[*1]

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Abstract The Applied Microbiology Section in the National Research Institute of Fisheries Science is nominated as a Microbial Culture Collections Sub-Bank of the Gene Bank in the Fisheries Research Agency (FRA) and keeps a collection of fifty strains of bacteria and yeast as of May 2002, that are in preparation to be utilized as open cultures. Microbiological characterization was carried out with these collections to assist future culture utilization. The study was conducted using several commercial kits including NF-18 test (for gram negative microorganisms), NF-20 test (for gram positive microorganisms), API50CH test, and API zym20 test in addition to conventional phenotypic characterization. The partial nucleotide sequences of 16S rRNA gene (for bacteria) and 18S rRNA gene (for yeast) were also determined and submitted to DDBJ. Based on all the obtained results, tentative identification was conducted for the current fifty FRA collections.

Key Words: characterization, FRA culture collection, marine microorganism, microorganism Sub-Bank of FRA

独立行政法人水産総合研究センター（Fisheries Research Agency, FRA）が実施する水産生物遺伝資源保存事業は，藻類・微細藻類サブバンク，微生物サブバンク，およびDNAサブバンクから構成される。中央水産研究所利用化学部応用微生物研究室は，本事業において微生物サブバンクに所属し，海洋微生物の収集・保存を担当している。2002年5月から約1年間かけて応用微生物研究室に保存されている菌株を整理し，産業利用の観点から重要と考えられる50菌株を選択し，2003年3月にベースコレクションとして登録した。本事業におけるベースコレクションとは，保存に値する菌株として位置づけられた菌株のことであり，今回登録された菌株には，マコンブ葉体分解細菌，海藻発酵菌（乳酸菌および酵母），および中国淡水魚醤油から分離された魚醤油乳酸菌等が含まれている。これらのベースコレクションを外部機関への分譲株として利用していくことについては，現在水産総合研究センターにおいて制度的検討が行われている段階であるが，将来菌株の分譲制度が開始した場合を想定して，菌株に関する性状分析データを充実させておくことがサブバンクに求められている。このような背景から上記50菌株のベースコレクションについて分類学的性状試験を実施

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した。性状試験の実施にあたっては，水産遺伝資源保存事業の担当者にかかわらず，データの品質基準が変わらずに継承性をもって試験が実施できること，また必要な場合には簡便に追試が実施できることが重要となる。このことから，できるだけ市販の同定キットを使用し，簡便なスキームで性状試験を実施することをこころがけた。具体的な試験項目としては，全菌株について基本性状試験を実施した後，グラム陰性の菌株にはNF-18（日水製薬）試験を，グラム陽性の菌株（酵母，乳酸菌，*Bacillus*属細菌）については，EB-20（日水製薬）試験を実施した。また同じく市販キットによりAPI 50CH（ビオメリュー）試験およびAPI zym 20（ビオメリュー）試験を実施した。さらに細菌株については，16S rRNA遺伝子（16S rDNA），酵母株については，18S rRNA遺伝子（18S rDNA）の部分塩基配列をそれぞれ決定し，これらの結果に基づいて暫定的な分類同定を行った。キット試験は，元来非好塩性微生物を対象にして開発されているため，好塩性を特徴とする海洋微生物の試験に適用する場合には試験方法を一部改変すべきと考えられるが，その実施方法についての情報は乏しい。そこでキット試験を実際に行って気のついた点を結果と考察の部分に記載した。

材料と方法

対象とした水産微生物遺伝資源

2002年5月時点で中央水産研究所利用化学部応用微生物研究室（水産生物遺伝資源保存事業微生物サブバンク海洋微生物部門）に収集保存されてある全微生物410菌株のうち，生存及び純粋性の確認された菌株の中から，これまで明らかにされている性状を参考に産業利用の観点から重要と考えられる50菌株を選定し，試験の対象とした（Table 1）。Table 1には推奨される培養条件も合わせて示した。

基礎性状試験

グラム染色試験は，市販染色試薬（日水製薬，フェイバーGセット）を使用した。細胞の形態，運動性，および胞子の形成の有無は，菌体を光学顕微鏡で観察して記載した。鞭毛は，常法に従って酢酸ウラニウムでネガティブ染色し（門田，多賀，1985），透過型電子顕微鏡（日本電子株式会社，JEM-1200EXⅡ）で観察した。OF試験，カタラーゼ，オキシダーゼ試験の実施は，常法に従った（長谷川，1990；門田，多賀，1985；河合ら，1988）。試験の実施にあたり菌体の培養は，特に記載がなければ20℃で実施した。

増殖温度試験は，菌体番号FRA000001～FRA000032についてはMarine Broth培地(Difco)を，FRA000033～FRA000036についてはMRS培地（Merck）を，FRA000037～FRA000040については7％(w/v) NaCl添加MRS培地（pH7.8に改変）を，FRA000041～FRA000044については標準培地（ペプトン5.0g，酵母エキス2.5g，グルコース1.0g/L，pH7.0）を，FRA000045～FRA000050についてはサブロー培地（ペプトン10g，グルコース40g/L，pH5.9）を使用した。Marine Broth培地（Difco）の調製にあたっては，加熱融解後，GF/Cでろ過して，濁りをとった後,オートクレーブ処理することにより沈殿物のない培地として使用した。菌体1白金耳を培地に接種し，5日間培養する過程で経時的に濁度を目視観察して増殖の有無を判断した。

増殖塩濃度試験は，FRA000001～FRA000032については改変標準培地（pH7.6に改変）を基本にNaClを各濃度で添加した培地を，FRA000033～FRA000036についてはMRS培地を，FRA000037～FRA000040については改変MRS培地（pH7.8に改変）を，FRA000041～FRA000044については標準培地を，FRA000045～FRA000050ついてはサブロー培地を基本として，NaClを各濃度で添加した液体培地を使用した。菌体1白金耳を培地に接種し，5日間培養する過程で経時的に濁度を目視観察して増殖の有無を判断した。

市販キットによる性状分析

キット試験の方法は，添付資料に従い，必要な場合は一部改変して実施した。また菌体は，特に記載がなければ20℃で培養した。グラム陰性の菌株にはNF-18（日水製薬）試験を，グラム陽性の菌株（酵母，乳酸菌，*Bacillus*属細菌）については，EB-20(日水製薬)試験を実施した。一部の株を除いてAPI 50CH（ビオメリュー）試験を，また全ての菌株についてAPI zym 20（ビオメリュー）試験を実施した。

NF-18及びEB-20試験に使用する菌体試料は，酵母，乳酸菌，*Bacillus*属細菌，グラム陰性菌について，それぞれサブロー寒天培地（SBR，日水製薬），MRS寒天培地（1.5%寒天添加MRS培地），標準寒天培地（SMA，日水製薬），Marine Broth寒天培地（MA）で培養して得た新鮮な菌体を，平板上より白金耳で掻き採り，キットに添付された菌体調製液に懸濁して使用した。グラム陰性菌については，塩要求性を考慮して，添付されたNF-18ブイヨン2mLに滅菌4％（w/v）濃度NaCl水溶液1mLを加えた改変菌体調製液に菌体を懸濁させた後，これを100μLずつ接種した。魚醤油乳酸菌については同4％濃度NaCl水溶液の代わりに滅菌30％濃度NaCl水溶液1mLを使用した。NF-18及びEB-20

Table 1. List of 50 strains tested in the present study

Strain No.	Strain symbol	Characeristics	Isolation source	Recommended culture media	Culture temp.(℃)	Ref.*1
FRA000001	AR03	Able to decompose *Laminaria* thallus	Seawater, at Yokosuka	Marine Agar	20	1
FRA000002	AR06	Able to decompose *Laminaria* thallus	Seawater, at Yokosuka	Marine Agar	20	1,2,3,4, 5,6,7,8
FRA000003	EN02	Able to decompose *Laminaria* thallus	Seawater, at Shimizu	Marine Agar	20	1
FRA000004	EN06	Able to decompose *Laminaria* thallus	Seawater, at Shimizu	Marine Agar	20	1
FRA000005	EN09	Able to decompose *Laminaria* thallus	Seawater, at Shimizu	Marine Agar	20	1
FRA000006	EN10	Able to decompose *Laminaria* thallus	Seawater, at Shimizu	Marine Agar	20	1
FRA000007	HA02	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1, 8
FRA000008	HA03	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000009	HA04	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000010	HA5a	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000011	HA5b	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000012	HA06	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000013	HA07	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000014	HA08	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000015	HA09	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000016	HA10	Able to decompose *Laminaria* thallus	Seawater, at Hakodate	Marine Agar	20	1
FRA000017	HO02	Able to decompose *Laminaria* thallus	Seawater, at Kushiro	Marine Agar	20	1
FRA000018	HO08	Able to decompose *Laminaria* thallus	Seawater, at Kushiro	Marine Agar	20	1
FRA000019	KY01	Able to decompose *Laminaria* thallus	Seawater, at Yosa	Marine Agar	20	1
FRA000020	KY09	Able to decompose *Laminaria* thallus	Seawater, at Yosa	Marine Agar	20	1
FRA000021	NA08	Able to decompose *Laminaria* thallus	Seawater, at Saeki	Marine Agar	20	1
FRA000022	SE02	Able to decompose *Laminaria* thallus	Seawater, at Hirado	Marine Agar	20	1
FRA000023	SE04	Able to decompose *Laminaria* thallus	Seawater, at Hirado	Marine Agar	20	1
FRA000024	SU01	Able to decompose *Laminaria* thallus	Seawater, at Shimonoseki	Marine Agar	20	1
FRA000025	YO02	Able to decompose *Laminaria* thallus	Seawater, at Watarai	Marine Agar	20	1
FRA000026	YO04	Able to decompose *Laminaria* thallus	Seawater, at Watarai	Marine Agar	20	1
FRA000027	HA-22	Able to decompose *Laminaria* thallus	Seawater, Hakodate	Marine Agar	20	9
FRA000028	HA-23	Able to decompose *Laminaria* thallus	Seawater, Hakodate	Marine Agar	20	9
FRA000029	HA-91	Able to decompose *Laminaria* thallus	Seawater, Hakodate	Marine Agar	20	9
FRA000030	KU-5	Able to decompose *Laminaria* thallus	Seawater, Kushiro	Marine Agar	20	9
FRA000031	KU-42	Able to decompose *Laminaria* thallus	Seawater, Kushiro	Marine Agar	20	9
FRA000032	KU-71	Able to decompose *Laminaria* thallus	Seawater, Kushiro	Marine Agar	20	9
FRA000033	B5201	Able to ferment seaweed	Fermented *Ulva* spp.	MRS, BCP Plate Count Agar	20	10
FRA000034	B5202	Able to ferment seaweed	Fermented *Ulva* spp.	MRS, BCP Plate Count Agar	20	
FRA000035	B5406	Able to ferment seaweed	Fermented *Ulva* spp.	MRS, BCP Plate Count Agar	20	
FRA000036	B5409	Able to ferment seaweed	Fermented *Ulva* spp.	MRS, BCP Plate Count Agar	20	
FRA000037	FSB101	Dominant in Silver carp-fish source	Silver carp-fish sauce	Modified MRS(7%NaCl, pH7.8)	20	11
FRA000038	FSB105	Dominant in Silver carp-fish source	Silver carp-fish sauce	Modified MRS(7%NaCl, pH7.8)	20	11
FRA000039	FSB401	Dominant in Silver carp-fish source	Silver carp-fish sauce	Modified MRS(7%NaCl, pH7.8)	20	11
FRA000040	FSB404	Dominant in Silver carp-fish source	Silver carp-fish sauce	Modified MRS(7%NaCl, pH7.8)	20	11
FRA000041	A0201	Dominant in fermented *Undaria*	Fermented *Undaria*	Standard Method Agar	20	
FRA000042	A1102	Dominant in fermented *Undaria*	Fermented *Undaria*	Standard Method Agar	20	
FRA000043	A1501	Dominant in dried *Undaria* products	*Undaria* pinnatifida (Commercial product)	Standard Method Agar	20	
FRA000044	A1513	Dominant in dried *Undaria* products	*Undaria* pinnatifida (Commercial product)	Standard Method Agar	20	
FRA000045	Y4101	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	
FRA000046	Y5201	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	10
FRA000047	Y5206	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	10
FRA000048	Y5207	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	
FRA000049	Y5317	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	
FRA000050	Y5318	Able to ferment seaweed	Fermented *Ulva*	Sabouraud Agar	20	

*1 1, Uchida and Nakayama (1993); 2, Uchida (1995); 3, Uchida; (1996); 4, Uchida and Kawamura (1995); 5, Uchida and Numaguchi (1996); 6, Uchida *et al.* (1997a); 7, Uchida *et al.* (1997b); 8, Uchida *et al.* (2002); 9, Uchida *et al.* (1995); 10, Uchida and Murata (2002); 11, Uchida *et al.* (2001).

は，20℃で5日間培養する間毎日1回観察して判定した。

API 50CH試験に使用する菌体試料は，菌体番号FRA000001，FRA000003，FRA000005～FRA000007，FRA000017～FRA000022，FRA000024については培養液API 50CH E/B（ビオメリュー）5 mLに，滅菌30%濃度NaCl水溶液0.5mLを添加して調製した改変培養液に，FRA000033～FRA000036については，API 50CHL（ビオメリュー）に，FRA000037～FRA000040については，API 50CHL 5 mLに滅菌30%濃度NaCl水溶液2 mLを添加して調製した改変培養液に，FRA000041～FRA000050についてはAPI 50CH E/B（ビオメリュー）に，それぞれ菌体をマクファーランド濁度2の濃度で懸濁させたものを120 μLずつ接種し，滅菌流動パラフィンを2滴添加した。API 50CHは，20℃で5日間培養後判定した。

API zym 20に使用する菌体試料は，上記寒天平板で培養して得た新鮮な菌体を白金耳で掻き採り，滅菌液1.5mLに105～107cells/mLの濃度（肉眼観察でやや濁りを感じる濃度）で懸濁後，遠心（10,000rpm，10min）洗浄後，同滅菌液に再懸濁したものを65 μLずつ接種した。滅菌液は，酵母，乳酸菌，*Bacillus*属細菌については，0.85%濃度NaCl水溶液を，グラム陰性細菌については2.5%濃度NaCl水溶液を，魚醤油乳酸菌については7%濃度NaCl水溶液を使用した。API zym 20は，20℃で24時間培養後，判定した。一部の菌株については，35℃で4時間培養して判定結果を比較した。また，一部の菌株については，マコンブ液体培地（0.1gマコンブ粉末，9 mL自然海水，1 mL蒸留水，オートクレーブ滅菌）入り試験管に菌体を1白金耳接種し，20℃，5rpmで3日間回転培養（タイテック，RT-550）して得た菌体画分および培養液上清画分についてもAPI zym 20試験を実施した。菌体画分および培養液上清画分の調製は，培養液を最初に軽く遠心処理（1,000rpm，10秒）してマコンブ粒子を取り除いた上清について，遠心処理（10,000rpm，10分）して行い，得た菌体を滅菌液に懸濁したものを菌体画分，このときの上清を培養液上清画分とした。

細菌の16S rDNAおよび酵母の18S rDNAの部分配列の決定

寒天平板上のコロニーを採取し，20 μLのTEバッファに懸濁した後，94℃15分加熱して得た上清1 μLを鋳型DNAとし，ユニバーサルプライマーセット27F（5'-AGAGTTTGATCCTGGCTCAG-3'）および1492R（5'-TACGGTTCCTTGTTACGACTT-3'）（Weisburg *et al.*, 1991）を使用してPCR増幅を行い，約1.5kbの16S rDNA断片を得た。27Fプライマーを使用してダイレクトシークエンシング法によりV1-V3領域を含む先頭の約300bpの塩基配列を決定し，DDBJに登録した。一部の菌株についてはほぼ全長域の塩基配列を決定して，DDBJに登録した。また既に塩基配列が決定されている菌株については，アクセッション番号を引用して記載した。決定された塩基配列をもとにBLASTにより統合データベース（DDBJ/EMBL/GenBank）から最近縁種を検索し，この検索結果と一般性状試験及びキット試験の結果をもとに総合的に判断して，供試菌株の分類同定を行った。酵母の場合には，ユニバーサルプライマーセットP1（5'-ATCTGGTTGATCCTGCCAGT-3'）およびNS8（5'-TCCGCAGGTTCACCTACGGA-3'）（Suzuki and Nakase, 1999）を使用してPCR増幅を行い，約1.8kbの18S rDNA断片を得たのち同様にプライマーNS8を使用して，末端の約300bpの塩基配列を決定し，DDBJへ登録した。酵母についてもBLASTによる最近縁種の検索の結果および既知の性状データを基に分類同定を行った。

結果と考察

基礎性状試験

Table 2に基礎性状試験の結果を示す。

NF-18試験およびEB-20試験

Table 3にNF-18試験の結果を，Table 4にEB-20試験の結果を示す。

API 50CH試験

Table 5にAPI 50CH試験の結果を示す。

海洋微生物の試験の実施に際しては，通常20℃ 2～3日程度の培養で陽性反応が観察できるが，徐々に陽性反応を示す場合もあったため，5日間以上経時的に観察して結果を判定した。FRA 000037株～FRA 000040株は，いずれも1年間室温下で発酵させた白蓮魚醤油試料から分離され，16S rRNA遺伝子の部分塩基配列からいずれも*Tetragenococcus halophilus*と同定された。FRA000037株とFRA000038株およびFRA000039株とFRA000040株は，それぞれ同じ発酵タンクから優占種として分離された経緯があるが，2菌株間の糖質資化性は，それぞれの場合ともいくつかの基質に対して異なっていた。FRA000008株～FRA000013株，FRA000015株～FRA000016株については，本試験を実施しなかったが，これらの菌株は他の性状がFRA000007株とほぼ一致することから，FRA000007株のAPI 50CH試験結果を参照データとして利用できるものと推察される。

Table 2. Phenotypic characteristics of the tested strains

Strain No.	Gram stain	Cell shape	Motility	Flagellar	Spore	OF	Oxidase	Catalase	Pigment	Growth temp. (℃)							NaCl conc. (%)			
										4	20	30	35	40	45	50	0	2.5	5	10
FRA000001	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000002	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	-	NT	NT	-	+	-	NT
FRA000003	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000004	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000005	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000006	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	+	NT
FRA000007	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000008	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000009	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000010	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000011	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000012	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	-	NT	NT	NT	-	+	-	NT
FRA000013	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000014	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000015	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000016	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000017	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000018	-	Short rod	+	Polar	-	O	+	+	-	-	+	-	-	NT	NT	NT	-	+	-	NT
FRA000019	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000020	-	Short rod	+	NT	-	-	-	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000021	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000022	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000023	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000024	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000025	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	+	NT
FRA000026	-	Short rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	+	NT
FRA000027	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000028	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000029	-	Rod	+	Polar	-	O	+	+	-	-	+	+	+	NT	NT	NT	-	+	-	NT
FRA000030	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000031	-	Rod	+	Polar	-	O	+	+	-	+	+	+	+	NT	NT	NT	-	+	-	NT
FRA000032	-	Rod	-	-	-	O	+	+	Yellow	-	+	+	+	NT	NT	NT	-	+	-	NT
FRA000033	+	Rod	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	NT
FRA000034	+	Rod	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	NT
FRA000035	+	Rod	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	NT
FRA000036	+	Rod	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	NT
FRA000037	+	Short Rod, Tetrads or pairs	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	+
FRA000038	+	Short Rod, Tetrads or pairs	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	+
FRA000039	+	Short Rod, Tetrads or pairs	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	+
FRA000040	+	Short Rod, Tetrads or pairs	-	-	-	F	-	-	-	NT	+	+	+	+	NT	NT	-	+	+	+
FRA000041	+	Rod	+	NT	+	-	-	+	-	NT	+	+	+	+	+	-	+	+	+	NT
FRA000042	+	Rod	+	NT	+	-	-	+	-	NT	+	+	+	+	+	-	+	+	+	NT
FRA000043	+	Rod	+	NT	+	-	-	+	-	NT	+	+	+	+	+	-	+	+	+	NT
FRA000044	+	Rod	+	NT	+	-	-	+	-	NT	+	+	+	+	+	-	+	+	+	NT
FRA000045	+	Spherical~slightly oval	-	-	+	F	-	+	-	NT	+	+	+	+	NT	NT	+	+	+	-
FRA000046	+	Spherical~slightly oval	-	-	+	F	-	+	-	NT	+	+	+	+	NT	NT	+	+	+	-
FRA000047	+	Oval	-	-	-	F	-	+	-	NT	+	+	+	-	NT	NT	+	+	+	-
FRA000048	+	Oval	-	-	-	F	-	+	-	NT	+	+	+	-	NT	NT	+	+	+	-
FRA000049	+	Spherical~slightly oval	-	-	NT	F	-	+	-	NT	+	+	+	+	NT	NT	+	+	+	-
FRA000050	+	Spherical~slightly oval	-	-	NT	F	-	+	-	NT	+	+	+	+	NT	NT	+	+	+	-

NT: Not tested

Table 3. Results of the NF-18 tests

Strain No.	GLU	FRU	MLT	GAL	XYL	MAN	SUC	LAC	ESC	URE	CIT	ONPG	LDC	ADH	ODC	IND	NIT	GEL
FRA000001	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
FRA000002	+	+	+	+	+	+	+	+	+	-	+	+	+	±	+	-	-	+
FRA000003	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+
FRA000004	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+
FRA000005	+	+	+	+	-	+	+	+	+	±	-	+	-	-	+	-	+	
FRA000006	+	+	+	+	-	-	+	+	-	+	-	-	+	+	+	-	-	+
FRA000007	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-
FRA000008	+	+	+	+	+	+	+	+	+	+	-	+	±	±	+	-	-	-
FRA000009	+	+	+	+	+	+	+	+	+	+	-	+	+	±	+	-	+	-
FRA000010	+	+	+	+	+	+	+	+	+	-	-	+	+	±	±	-	+	-
FRA000011	+	+	+	+	+	+	+	+	+	+	-	+	±	±	+	-	+	-
FRA000012	+	+	+	+	+	+	+	+	+	-	-	+	+	±	±	-	+	-
FRA000013	+	+	+	+	+	+	+	+	+	+	-	+	±	±	+	-	+	-
FRA000014	+	+	+	+	+	-	+	+	+	+	-	-	±	±	+	-	-	+
FRA000015	+	+	+	+	+	+	+	+	+	-	-	+	±	+	±	-	+	-
FRA000016	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	
FRA000017	+	+	+	+	-	+	+	+	+	-	-	+	±	+	+	-	+	+
FRA000018	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	-	+	+
FRA000019	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	-	+	+
FRA000020	+	+	+	+	+	+	+	+	-	-	+	+	±	+	+	-	-	-
FRA000021	+	+	+	+	+	+	+	+	+		-	+	-	-	-	-	+	-
FRA000022	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+
FRA000023	+	+	+	+	-	+	+	+	+	+	-	+	±	±	+	-	+	+
FRA000024	+	+	+	+	+	+	+	+	+	+	-	+	+	±	+	-	+	-
FRA000025	+	-	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	+
FRA000026	+	±	+	+	-		+	+	-	+	-	-	+	+	+	-	-	+
FRA000027	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+
FRA000028	+	+	+	+	+	+	+	+	+	-	-	+	±	+	+	-	+	+
FRA000029	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+
FRA000030	+	+	+	+	+	+	+	+	+	-	-	+	+	±	+	-	+	+
FRA000031	+	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	+	+
FRA000032	+	+	+	+	+	+	+	+	+	+	-	+	±	+	—	-	-	-

+; positive, ±; not clear, -; negative. GLU; glucose test, FRU; fructose test, MLT; maltose test, GAL; galactose test, XYL; xylose test, MAN; mannitol test, SUC; sucrose test, LAC; lactose test, ESC; esculin test, URE; urease test, CIT; citrate test, ONPG; ortho-nitrophenyl-β-D-galactopyranoside test, LDC; lysine decarboxylase test, ADH; arginine dehydrolase test, ODC; ornithine decarboxylase test, IND; Indole test, NIT; nitrase test, GEL; gelatin test. Test plates were cultured for 5 days at 20℃.

Table 4. Results of the EB-20 tests

Strain No.	H2S	ESC	PPA	IND	VP	CIT	LDC	ADH	ODC	ONPG	URE	MALO	ADO	INO	RAFF	RHA	SOR	SUC	MAN	ARA
FRA000033	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	±	+
FRA000034	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	±	±	+
FRA000035	-	+	-	-	+	-	-	-	-	+	-	-		-		±	+	+	+	-
FRA000036	-	+	-	-	+	-	-	-	-	+	-	-	-		-	±	+	+	+	
FRA000037	-	-	-	+	-	-		-	-	+	+	-	-	-	-	-	-	+	+	+
FRA000038	-	±	-	+	-	-	-	-	-	-	-	-		-	-	-	-	-	-	
FRA000039	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FRA000040	-	+	-	±		-	-	-	-	-	-	-		-	-	-	+	-	±	+
FRA000041	-	+	-	-	+	-	-	-	-	-		-	-	-	-	-	-	-		
FRA000042	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
FRA000043	-	+	-	-	+	-		-		-	+	-	-	-	-	-	-	-	-	-
FRA000044	-	+	-	-	-	-	-	-	-		-	-		-	-	-	-	-	-	-
FRA000045	-	+	-	-	+	-	-	-	-	-		-	+	-	+	+	+	+	+	+
FRA000046	-	+	-	-	+	-	-	-	-	-	-	-	+		+	+	+	+	+	+
FRA000047	-	+	-	-		-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
FRA000048	-	+	-	-	-		-		-	-		-	+	+	+	+	+	+	+	+
FRA000049	-	+	-	-	-	-		-	-	-	-	-	+		+	+	+	+	+	+
FRA000050	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+

+; positive, ±; not clear, -; negative. H2S; Hydrogen sulfide test, ESC; esculin test, PPA; phenylalanine test, IND; Indole test, VP; Voges Proskauer test, CIT; citrate test, LDC; lysine decarboxylase test, ADH; arginine dehydrolase test, ODC; ornithine decarboxylase test, ONPG; ortho-nitrophenyl-β-D-galactopyranoside test, URE; urease test, MALO; malonic acid test, ADO; adonite test; INO; inositol test, RAFF; raffinose test, RHA; rhamnose test, SOR; sorbitol test, SUC; sucrose test, MAN; mannite test, ARA; levo-arabinose test. Test plates were cultured for 5 days at 20℃.

Table 5. Results of the API 50CH tests

Strain No.	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	βMethyl-xyloside	Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	αMethyl-D-mannoside	αMethyl-D-glucoside	N Acetyl glucosamine	Amygdaline	Arbutine	Esculine	Salicine	Cellobiose	Maltose	Lactose	Melibiose	Saccharose	Trehalose	Inuline	Melezitose	D-Raffinose	Amidon	Glycogene	Xylitol	βGentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2keto-gluconate	5keto-gluconate	Medium
FRA000001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FRA000002	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	API50CHB/E (+2.7%NaCl)
FRA000003	-	-	-	-	-	-	-	-	-	+	-	→	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FRA000004	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	API50CHB/E (+2.7%NaCl)
FRA000005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	±	-	-	±	-	-	-	-	±	±	-	-	-	-	-	-	-	-	-	-	-	
FRA000006	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000007	-	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	±	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	±	±	-	-	-	±	+	API50CHB/E (+2.7%NaCl)
FRA000008	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	API50CHB/E (+2.7%NaCl)
FRA000009	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000010	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000011	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000012	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000013	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000014	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000015	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000016	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000017	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FRA000018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	+	-	±	±	-	-	+	-	-	-	-	±	±	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000019	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	±	±	-	-	-	-	-	-	-	±	±	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000020	±	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	±	±	±	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000021	+	-	-	±	±	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	-	-	+	±	+	+	±	+	±	±	-	-	-	-	±	-	-	+	-	+	+	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000022	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	API50CHB/E (+2.7%NaCl)
FRA000023	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	API50CHB/E (+2.7%NaCl)
FRA000024	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FRA000025	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	API50CHB/E (+2.7%NaCl)
FRA000026	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000027	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000028	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000029	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000030	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000031	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000032	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
FRA000033	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
FRA000034	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	±	API50CHL
FRA000035	-	-	-	-	+	-	-	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	API50CHL
FRA000036	-	-	-	-	+	-	-	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	API50CHL
FRA000037	+	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	API50CHL
FRA000038	+	+	-	+	-	+	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	API50CHL (+8.6%NaCl)
FRA000039	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	API50CHL (+8.6%NaCl)
FRA000040	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	API50CHL (+8.6%NaCl)
FRA000041	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	API50CHL (+8.6%NaCl)
FRA000042	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	±	±	-	-	-	API50CHB/E
FRA000043	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	±	+	-	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E
FRA000044	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	±	-	-	-	-	API50CHB/E
FRA000045	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	-	-	+	+	-	+	±	±	+	+	+	+	+	+	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	API50CHB/E
FRA000046	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	+	API50CHB/E
FRA000047	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	+	+	-	+	-	+	+	+	-	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	+	API50CHB/E
FRA000048	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	-	+	-	+	+	+	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	+	API50CHB/E
FRA000049	-	-	-	+	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	+	API50CHB/E
FRA000050	-	-	-	+	-	+	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	+	API50CHB/E

Production of acid from the above carbohydrates were observed. +; positive, ±; not clear, -; negative, NT; not tested. Cell suspension media were moified for testing halophilic microorganisms and the final salt concentrations were shown in parentheses. Test plates were cultured for 5 days at 20℃.

API zym20試験

Table 6にAPI zym20試験の結果を示す。

データを収集するにあたり, 測定条件について, FRA000001株, FRA000005株およびFRA000045株を対象として, 若干の検討を加えた。まず, 細菌の調製法について, 前者2株を被験株としてMarine Broth寒天培地で培養後菌体を掻きとって細胞懸濁液を調製した場合とマコンブ液体培地で培養後遠心処理により集菌して細胞懸濁液を調製した場合とで比較した。その結果, 2株の場合とも前者の方が酵素活性の陽性項目が多い傾向が認められた。またマコンブ液体培地で培養後遠心処理により集菌して得た細胞懸濁液と培養上清液とを被験試料として比較した。その結果, ほぼ同じ結果か, 幾分前者の方が陽性項目が多くなる傾向があった。反応温度条件については, 20℃24時間と35℃ 4時間とで比較した。その結果, 試料により若干陽性項目に違いがみられたが, 細胞懸濁液を被験試料とする場合に限っていえば, 20℃24時間反応の方が若干優れる傾向があった。以上の検討結果を踏まえ, 寒天平板培地で培養した菌体を掻きとって調製した菌体懸濁液を被験試料とし, 反応条件は20℃24時間として分析することを基本とした。なおFRA000037株～FRA000040株は, 増殖速度が遅いことを考慮して反応条件は20℃43時間とした。

16S rDNAおよび18S rDNAの部分配列に基づいた近縁種の検索と同定結果

各菌株について16S rDNAおよび18S rDNAの部分配列を決定し, DDBJに登録して得たアクセッション番号をTable 7に記載した。さらに決定された塩基配列をBLAST検索にかけ最近縁種を示すとともに, 最近縁種の塩基配列と相違する塩基数を示した。同定は, rDNAの部分塩基配列の情報と性状試験の結果から総合的に判断して行った。FRA000003株, FRA000004株, FRA000023株, FRA000024株は, 酸産生により判断した炭素源資化性試験において, マンノース陽性, フルクトース陽性であることから*Pseudoalteromonas haloplanctis*と同定された（Ivanova *et al.*, 2001)。FRA000005株は, 16S rDNAの後半861塩基の配列（AB106188）に基づいてデータベース上で検索した最近縁種は*Pseudoalteromonas citrea*であったが, マンノース陰性, ガラクトース陰性, トレハロース陰性であることから*Pseudoalteromonas carraginovora*と暫定的に同定した（Sawabe *et al.*, 2000)。FRA000017株～FRA000019株, FRA000022株, FRA000027株は, マンノース陽性, ガラクトース陽性, スクロース陽性, マルトース陽性, グルコネート陰性, トレハロース陰性であることから*Pseudoalteromonas elyakovii*と同定された（Sawabe *et al.*, 2000)。FRA000025株, FRA000026株は, マンノース陰性, ガラクトース陽性, フルクトース陰性, スクロース陽性, マルトース陽性であることから*Pseudoalteromonas tetraodonis*と同定された（Sawabe *et al.*, 2000)。FRA000028株, FRA000030株, FRA000031株は, Bergey's Manual（Baumann *et al.*, 1984）に記載された性状と比較して*Alteromonas macleodii*と同定された。乳酸菌については, Bergey's Manual（Kandler and Weiss, 1984）およびCollins *et al.*（1990）により記載された性状と比較してTable 7のとおり同定された。FRA000041株, FRA000043株は, Claus and Berkeley（1984）により記載された性状と比較して*Bacillus cereus*と同定された。FRA000046株, FRA000047株は, The Yeast（Kurtzman and Fell, 1998）に記載された性状と比較して*Debaryomyces hansenii* var. *hansenii*と同定された。残りの22株については, 今回の試験データだけでは種までの特定ができなかった。これらの株の種の同定については, より変異の大きい遺伝子の塩基配列の決定が必要と考えられた。

Table 6 Results of the API zym20 tests

Strain No.	Enzymes																			Test method		
	Alkali-phosphatase	Esterase (C4)	Esterase (C8)	Lipase (C14)	Leucine-allylamidase	Varine-Allylamidase	Cystein-allylamydase	Trypsin	Chymotrypsin	Acidic phosphatase	Naphtol-AS-BI-phosphahydrorase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α—Glucosidase	β-Glucosidase	N-Acetyl-β-Glucosaminidase	α—Mannosidase	α—Fucosidase	Sample preparation	Reacted conditions	
FRA000001	+	+	+	±	+	+w	±	-	-	+w	+w	-	±	-	+w	-	-	-	-	Cells	20℃	24h
	+	-	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	Cells (L)*1	20℃	24h
	±	-	±	-	±	-	-	-	-	±	+	-	-	-	-	-	-	-	-	Cells (L)*1	35℃	4h
	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	Culture sup.(L)*2	20℃	24h
	±	-	±	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	Culture sup.(L)*2	35℃	4h
FRA000002	+	+w	+w	+w	+	+	+	+	+	+	+	+	+	-	+	-	-	-	±	Cells	20℃	24h
FRA000003	+	+w	+w	+w	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000004	+	+w	+	+w	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000005	+	+w	+	+w	+	+	+	+	+	+	+	+w	+	-	+	-	-	-	-	Cells	20℃	24h
	+	±	+	-	+	-	-	-	-	+w	+	-	-	-	-	-	-	-	-	Cells (L)*1	20℃	24h
	+	±	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	Cells (L)*1	35℃	4h
	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	Culture sup.(L)*2	20℃	24h
	+	-	±	-	+	-	-	-	-	±	+	-	-	-	-	-	-	-	-	Culture sup.(L)*2	35℃	4h
FRA000006	+	+w	+	+w	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	Cells	20℃	24h
FRA000007	+	+	+	-	+	+	+	+w	+	+	+	+	+	-	+w	+w	-	-	-	Cells	20℃	24h
FRA000008	+	+	+	±	+	+	+	+w	+	+	+	+	+	±	+	+	-	-	-	Cells	20℃	24h
FRA000009	+	+	+	-	+	+	+	+w	+	+	+	+	+	-	+	+	-	-	-	Cells	20℃	24h
FRA000010	+	+	+	±	+	+	+	+w	+	+	+	+	+	-	+	+w	-	-	-	Cells	20℃	24h
FRA000011	+	+	+	-	+	+	+	+w	+w	-	+	+	+w	-	+w	+w	-	-	-	Cells	20℃	24h
FRA000012	+	+	+	±	+	+	+w	+w	+	+	+	+	+	-	+	+	-	-	-	Cells	20℃	24h
FRA000013	+	+w	+	-	+	+	+	+w	+w	+	+	+	+	-	+w	+w	-	-	-	Cells	20℃	24h
FRA000014	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	Cells	20℃	24h
FRA000015	+	+	+	±	+	+	+	+w	+	+	+	+	+	-	+	+	-	-	-	Cells	20℃	24h
FRA000016	+	+	+	-	+	+	+	+w	+	+	+	+	+	-	+	+	-	-	-	Cells	20℃	24h
FRA000017	+	+	+	±	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000018	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000019	+	+	+	-	+	+	+	+	+	+	+	+w	-	-	+	-	-	-	-	Cells	20℃	24h
FRA000020	+	+	+	-	+	±	±	-	-	+	+w	+w	+w	±	+	±	±	±	±	Cells	20℃	24h
FRA000021	+	+	+	±	+	+	+w	±	±	+w	+w	-	+	±	+	+w	-	-	-	Cells	20℃	24h
FRA000022	+	+	+	±	+	+	+	+	+	+	+	±	±	-	+	-	-	-	-	Cells	20℃	24h
FRA000023	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000024	+	+	+	-	+	+	+	+	+	+	+	+	+	-	±	-	-	-	-	Cells	20℃	24h
FRA000025	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	Cells	20℃	24h
FRA000026	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+w	-	-	-	-	Cells	20℃	24h
FRA000027	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	Cells	20℃	24h
FRA000028	+	+	+	+w	+	+	+	+	+	+	+	+	+	-	+	+w	-	-	-	Cells	20℃	24h
FRA000029	+	+	+	-	+	+	+	+w	±	+	+	-	-	-	-	-	-	-	-	Cells	20℃	24h
FRA000030	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+w	±	-	-	-	Cells	20℃	24h
FRA000031	+	+	+	+	+	+	+	+	+	+	+	±	±	-	-	+	-	-	-	Cells	20℃	24h
FRA000032	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	±	Cells	20℃	24h
FRA000033	+w	+	+	±	+	+	+	±	±	+	+	+	+	+	+	+	-	-	-	Cells	20℃	24h
FRA000034	+w	+	+w	+w	+	+	+	±	+	+	+	+	+	+	+	+	-	-	-	Cells	20℃	24h
FRA000035	+	+	+	+w	+	+	+	±	+	+	+	±	+	±	+	+w	+w	-	+	Cells	20℃	24h
FRA000036	+	+w	+	±	+	+	+w	±	+	+	+	-	+	-	+	+	+w	-	+	Cells	20℃	24h
FRA000037	+	+	+	±	+	-	-	-	±	+	+	-	-	-	-	-	-	-	-	Cells	20℃	43h
FRA000038	-	+w	+w	+w	+	+	+w	-	-	+w	+	-	-	-	+w	+	-	-	-	Cells	20℃	43h
FRA000039	-	-	-	-	+	+w	-	-	-	-	+	-	-	-	-	-	-	-	-	Cells	20℃	43h
FRA000040	-	-	-	-	+w	+w	-	-	-	+w	+w	-	-	-	-	+w	-	-	-	Cells	20℃	43h
FRA000041	-	+w	+w	-	+	±	-	-	±	+w	+w	-	-	-	+	-	-	-	-	Cells	20℃	24h
FRA000042	+	+	+	-	+	+	+	+w	+w	+	+w	+w	+w	-	+w	+w	+w	+w	+w	Cells	20℃	24h
FRA000043	+w	+	+	-	+	±	±	-	-	+	+w	-	-	-	+	-	-	-	-	Cells	20℃	24h
FRA000044	+	+	+	-	+	+	+	+	+	+	+w	+w	+w	-	+w	+w	+w	+w	+w	Cells	20℃	24h
FRA000045	+w	+	+	±	+	+	+w	-	-	+	+w	+w	-	-	+	+	-	-	-	Cells	20℃	24h
	+	+	+	±	+	+w	+w	±	±	+	+	-	-	-	-	-	-	-	-	Cells	35℃	4h
FRA000046	+	+	+	-	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	Cells	20℃	24h
FRA000047	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	Cells	20℃	24h
FRA000048	+	+	+	+w	+	+	+w	±	±	+	+	-	-	-	-	-	+	-	-	Cells	20℃	24h
FRA000049	+	+	+	±	+	+	+	+w	-	+	+	-	-	-	+	+	-	-	-	Cells	20℃	24h
FRA000050	+	+	+	±	+	+	+	±	±	+	+	-	-	-	+	+	-	-	-	Cells	20℃	24h

*1 The bacterial cells were prepared by being cultured on a medium containing 0.1g *Laminaria japonica*, 9ml seawater, and 1ml distilled water, collected by centrifuge, and suspended in 2.5% NaCl solution.

*2 Supernatant was collected by centrifuge after culturing bacterial cells on a medium containing 0.1g *L. ja ponica*, 9ml seawater, and 1ml distilled water.

+; positive, +w; positive but weak activity, ±; not clear, -; negative.

Table 7. (1/2) Identification of the 50 strains tested in the present study

Strain No.	16S/18S rDNA Accession No.	Most neighbor species	No. of different seq./determined seq. (bp)	Key characteristics (Utilization)	Concluded species	References
FRA000001	AB106185 AB106186	*Roseobacter sp.*	5/271		Alpha proteobacteria	
FRA000002	AB049728	*Pseudoalteromonas atlantica*	1/1424		*Pseudoalteromonas atlantica*	Uchida *et al.* 2002
FRA000003	AB105549	*Pseudoalteromonas haloplanktis*, *Pseudoalteromonas tetraodonis*	0/320	Mannose+ Galactose+ Fructose+ Trehalose-	*Pseudoalteromonas haloplanktis*	Ivanova *et al.*, 2001
FRA000004	AB105550	*Pseudoalteromonas haloplanktis*, *Pseudoalteromonas tetraodonis*	0/320	Mannose+ Galactose+ Fructose+ Trehalose-	*Pseudoalteromonas haloplanktis*	Ivanova *et al.*, 2001
FRA000005	AB106187	*Pseudoalteromonas atlantica*, *Pseudoalteromonas carrageenovora*	0/262	Mannose- Galactose- Trehalose-	*Pseudoalteromonas carraginovora*	Sawabe *et al.*, 2000
	AB106188	*Pseudoalteromonas citrea*	1/861			
FRA000006	AB106189	*Pseudoalteromonas elyakovii*, *Pseudoalteromonas agarovorans*	11/1463		*Pseudoalteromonas* sp.	
FRA000007	AB049729	*Glaciecola punicea*	37/1367		*Glaciecola* sp.	Uchida *et al.* 2002
FRA000008	AB106190	*Glaciecola pallidula*	21/320		*Glaciecola* sp.	
FRA000009	AB106191	*Glaciecola pallidula*	21/298		*Glaciecola* sp.	
FRA000010	AB106192	*Glaciecola pallidula*	21/313		*Glaciecola* sp.	
FRA000011	AB106193	*Glaciecola pallidula*	21/320		*Glaciecola* sp.	
FRA000012	AB106194	*Glaciecola pallidula*	21/312		*Glaciecola* sp.	
FRA000013	AB106195	*Glaciecola pallidula*	21/317		*Glaciecola* sp.	
FRA000014	AB106196	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas elyakovii*	0/317	Mannose- Lactose+	*Pseudoalteromonas* sp.	Sawabe *et al.*, 2000
	AB106197	*Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas tetraodonis*	0/558			
FRA000015	AB106198	*Glaciecola punicea*	13/231		*Glaciecola* sp.	
FRA000016	AB106199	*Glaciecola pallidula*	21/320		*Glaciecola* sp.	
FRA000017	AB106200	*Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens*	0/319	Mannose+ Galactose+ Sucrose+ Maltose+ Gluconate- Citrate- Trehalose-	*Pseudoalteromonas elyakovii*	Sawabe *et al.*, 2000
	AB106201	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas tetraodonis*	0/561			
FRA000018	AB106202	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas citrea*, *Pseudoalteromonas elyakovii*	0/319	Mannose+ Galactose+ Sucrose+ Maltose+ Gluconate- Citrate- Trehalose-	*Pseudoalteromonas elyakovii*	Sawabe *et al.*, 2000
	AB106203	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas atlantica*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas tetraodonis*	0/316			
FRA000019	AB106204	*Pseudoalteromonas atlantica*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens*	0/319	Mannose+ Galactose+ Sucrose+ Maltose+ Gluconate- Citrate- Trehalose-	*Pseudoalteromonas elyakovii*	Sawabe *et al.*, 2000
	AB106205	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas tetraodonis*	0/558			
FRA000020	AB106206	*Halomonas marina*	1/296		*Halomonas* sp.	
FRA000021	AB106207	*Marinomonas protea*	10/305		*Gamma proteobacteria*	
FRA000022	AB106208	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas atlantica*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas tetraodonis*	0/220	Mannose+ Galactose+ Sucrose+ Maltose+ Gluconate- Citrate+ Trehalose-	*Pseudoalteromonas elyakovii*	Sawabe *et al.*, 2000
FRA000023	AB106209	*Pseudoalteromonas haloplanktis*, *Pseudoalteromonas tetraodonis*	0/296	Mannose+ Fructose+	*Pseudoalteromonas haloplanktis*	Ivanova *et al.*, 2001
FRA000024	AB106210	*Pseudoalteromonas haloplanktis*, *Pseudoalteromonas tetraodonis*	0/315	Mannose+ Fructose+	*Pseudoalteromonas haloplanktis*	Ivanova *et al.*, 2001

Table 7. (2/2)

Strain No.	16S/18S rDNA Accession No.	Most neighbor species	No. of different seq./determined seq. (bp)	Key characteristics (Utilization)	Concluded species	References
FRA000025	AB106211	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas tetraodonis*	0/223	Mannose- Galactose+ Fructose- Sucrose+ Maltose+	*Pseudoalteromonas tetraodonis*	Ivanova *et al.*, 2001
FRA000026	AB106212	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas tetraodonis*	0/227	Mannose- Galactose+ Fructose- Sucrose+ Maltose+	*Pseudoalteromonas tetraodonis*	Ivanova *et al.*, 2001
FRA000027	AB106213	*Pseudoalteromonas agarovorans*, *Pseudoalteromonas citrea*, *Pseudoalteromonas distincta*, *Pseudoalteromonas elyakovii*, *Pseudoalteromonas nigrifaciens*,	0/277	Mannose+ Galactose+ Sucrose+ Maltose+ Citrate-	*Pseudoalteromonas elyakovii*	Sawabe *et al.*, 2000
FRA000028	AB106215	*Alteromonas macleodii*	0/298		*Alteromonas macleodii*	Baumann *et al.*, 1984
FRA000029	AB106216	*Glaciecola pallidula*	22/307		*Glaciecola* sp.	
FRA000030	AB106217	*Alteromonas macleodii*	0/303		*Alteromonas macleodii*	Baumann *et al.*, 1984
FRA000031	AB106218	*Alteromonas macleodii*	0/305		*Alteromonas macleodii*	Baumann *et al.*, 1984
FRA000032	AB106219	*Cytophaga fucicola*	0/105		*Cytophaga* sp.	
FRA000033	AB070607	*Lactobacillus brevis*	0/1475		*Lactobacillus brevis*	Kandler and Weiss, 1984
FRA000034	AB106341	*Lactobacillus brevis*	0/326		*Lactobacillus brevis*	Kandler and Weiss, 1984
FRA000035	AB070609	*Lactobacillus casei*	0/1488		*Lactobacillus casei*	Kandler and Weiss, 1984
FRA000036	AB106342	*Lactobacillus casei*	0/328		*Lactobacillus casei*	Kandler and Weiss, 1984
FRA000037	AB041347	*Tetragenococcus halophilus*	6/1491		*Tetragenococcus halophilus*	Collins *et al.*, 1990
FRA000038	AB106343	*Tetragenococcus halophilus*	0/316		*Tetragenococcus halophilus*	Collins *et al.*, 1990
FRA000039	AB041349	*Tetragenococcus halophilus*	5/1495		*Tetragenococcus halophilus*	Collins *et al.*, 1990
FRA000040	AB106344	*Tetragenococcus halophilus*	1/320		*Tetragenococcus halophilus*	Collins *et al.*, 1990
FRA000041	AB106345	*Bacillus cereus*	0/1470		*Bacillus cereus*	Claus and Berkeley., 1984
FRA000042	AB106346	*Bacillus sphaericus*	12/1413		*Bacillus sp.*	
FRA000043	AB106347	*Bacillus cereus*	0/258		*Bacillus cereus*	Claus and Berkeley., 1984
FRA000044	AB106348	*Bacillus sphaericus*	9/329		*Bacillus* sp.	
FRA000045	AB106349	*Debaryomyces hansenii var. hansenii*	2/1752		*Debaryomyces hansenii* var. *hansenii*	Kurtzman and Fell, 1998
FRA000046	AB070854	*Debaryomyces hansenii var. hansenii*	2/1752		*Debaryomyces hansenii* var. *hansenii*	Kurtzman and Fell, 1998
FRA000047	AB070855	*Candida zeylanoides*	8/1750		*Candida* sp.	
FRA000048	AB106350	*Candida zeylanoides*	8/1750		*Candida* sp.	
FRA000049	AB106351	*Debaryomyces hansenii* var. *fabryi*	6/1752		*Debaryomyces* sp.	
FRA000050	AB070856	*Debaryomyces hansenii* var. *fabryi*	6/1752		*Debaryomyces* sp.	

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博士号論文

Studies on genetic management of hatchery broodstock in relation to the stock enhancement of Japanese flounder *Paralichthys olivaceus*[*1]

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Abstract From the viewpoint of genetic conservation, this study presents application of molecular genetic markers to the population genetics studies of Japanese flounder *Paralichthys olivaceus* in order to propose several broodstock management options related to the stock enhancement.

A $(CA)_n$-microsatellite enriched library of Japanese flounder was constructed using an efficient method to isolate microsatellite DNA regions, from which 16 PCR primer pairs to amplify microsatellites with a high amplification effectiveness were developed. Genetic variability of these loci varied depending on the locus, and all but one of the 16 loci conformed to Hardy-Weinberg's equilibrium (HWE). Mendelian inheritability of these loci was verified in five experimental families of Japanese flounder excepting one instance. The locus at which the significant HWE departure was observed had unstable and unreproducible PCR amplification of a particular allele in one family. These results suggest that 15 out of the 16 microsatellites are viable for further applications to the wide range of the Japanese flounder population genetics studies.

Genetic variability and structure maintained in wild Japanese flounder populations (seven populations) were assessed by using 11 microsatellites coupled with the nucleotide sequences of mitochondrial DNA (mtDNA) control region to outline the genetic stock management units. The 11 microsatellites examined showed considerable polymorphisms in all populations, and the section of mtDNA sequences examined was highly variable. Assessment of the genetic structure based on the 11 microsatellites revealed that 11 population pairs in 21 possible pairwise comparisons yielded significant genetic heterogeneities in terms of allele frequency distributions and/or pairwise F_{ST} values, and an overall F_{ST} value provided evidence of the genetic structuring among the seven populations, although these inter-populational genetic heterogeneities were not necessarily supported by the results of mtDNA sequencing analysis. According to the population relationships estimated from the phylogenetic trees based on the genetic distances (D_A and D_{ST}) and the results of several hierarchical AMOVA analyses, which were calculated on the basis of the microsatellite data, the seven populations were possibly assigned into three groups consistent with the oceanogeographic positions of the populations. Although the level of the genetic differentiation between the groups was weak, the genetic management of wild flounder should be applied along with the group definitions so as to preserve the currently detectable genetic stock units.

Level of genetic variability maintained in currently available hatchery strains was compared with that of wild populations. Three Japanese flounder hatchery strains were

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screened by means of microsatellite and mtDNA sequencing analysis, and both the number of microsatellite alleles and mtDNA haplotypes, and mtDNA haplotype diversity showed marked reductions in the hatchery strains relative to the comparable three wild populations.

Pedigree structure of a hatchery strain was detailed in order to illustrate a skewed contribution of broodstock parents to the next generation, which may possibly cause a reduced effective population size, by using microsatellite profiling technique. The pedigrees of offspring including 113 individuals of larvae sampled within 24 hours after hatching, 216 individuals of one-month-old, and 407 individuals of four-month-old were unambiguously identified. The contribution of candidate broodstock parents (18 individuals) to the next generation was highly skewed as one male of the six males used monopolized the contribution to the offspring pool, and a half of the females (6 of 12 females) did not produce any offspring. The contribution of one family to the released fish, which were culled for larger size in total length, was significantly higher, while those of other two families were lower, suggesting that culling operations for particular traits might cause a large family size variance of released fish through selection in favor of a few family lineages. Culling operations for particular traits thus should be avoided as much as possible to minimize the risk of unexpected pedigree structure to be a possible cause of the reduced effective size.

Possible way to retard loss of genetic variations in closed and non-pedigreed captive populations of Japanese flounder was explored by using a microsatellite-based kinship estimator (r_{xy} statistic). The difference of pairwise r_{xy} values among and between full-sib, half-sib, and unrelated categories in a hatchery strain was highly significant, suggesting that the r_{xy} statistic would have a high utility to discriminate kinships. Minimal kinship (*mk*) selection approach based on the r_{xy} statistic, which is a breeding strategy to minimize loss of genetic variability in captive populations, turned out to be effective in retention of both allelic diversity and gene diversity; this strategy however did not necessarily select unrelated fish as parents to create the next generation. It should thus be necessary to preclude the mating between highly related individuals in the selected fish by using the r_{xy} statistic as a kinship indicator so as to minimize the risk of inbreeding.

From the results presented above, the present author discusses here several hatchery management options for further practical hatchery operations to minimize the genetic impact of stocking practice to the wild Japanese flounder populations.

Key words: Japanese flounder, Genetic markers, Population genetics, Conservation genetics, Stock enhancement

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Chapter 1. General introduction

The Japanese flounder *Paralichthys olivaceus* is an important component of coastal fishery resources in Japan, and is heavily exploited due to its highly commercial significance. Market landings of this species have not been greatly increased over the last decade (Anon, 2001) in spite of extensive fishery management effort such as regulations to counteract over-fishing, and thus a major interest has been directed toward the exploitable resource enhancement by means of stocking of hatchery-reared fish into the natural environment. The hatchery production of Japanese flounder was successfully developed in 1965, and stocking practices of flounder juveniles were embarked some 30 years ago (Furusawa, 1997). With the increase of flounder seedlings production, this species has become the most important target in the stock enhancement programs running in Japan (Anon, 2000); however the extent of stocking effects on the biomass and the ecological, biological, and genetic impacts of stocked fish to the wild populations have not been clearly ascertained. The hatchery fish are usually reared in a protected environment and thus exposed to different constraints from those experienced in the natural environment, which may possibly affect to the survival and growth of hatchery-produced fish. The qualitative disadvantages of hatchery-based fish in several traits have been a growing concern: abnormalities in pigmentation (reviewed in Seikai, 1997) and in ossification (Hosoya and Kawamura, 1996) have been observed frequently in hatchery fish, and the differences of feeding behavior between hatchery and wild fish were reported (Furuta *et al.*, 1997; Furuta, 1998a; Furuta, 1998b). These findings indicate that further massive stocking of hatchery-based fish into the natural environment may have the potential to cause harmful effects upon the wild fish populations, possibly resulting in the reduction of the resource mass.

The potential genetic impact of stocking practices to the wild fish populations also has been a focus of attention. This is because most hatchery strains typically show reduced genetic variations, which may possibly result in the loss of disease resistance, and in the reduction of the population's ability to adapt to a changing environment (Ryman and Stahl, 1980; Allendolf and Phelps, 1980; Allendorf and Ryman, 1987). A large scale and consistent stocking of hatchery-based fish will increase a short-term exploitation of resources; but, at the same time, this may however have potential detrimental genetic impacts on the wild populations (Allendorf and Ryman, 1987), as the reduction of genetic variability in salmonid fishes has been shown to cause harmful effects upon several traits such as mortality and growth rate (*e.g.*, Gjerde *et al.*, 1983; Kincaid, 1983; Su *et al.*, 1996). It is very likely that the loss of genetic variations in hatchery fish would be due to the limited number of effective parents or the occurrence of inbreeding events when the hatchery strains were founded. Therefore, hatchery operations should be made advertently, and a central tenet of a stock enhancement program is thus the notion that the genetic condition of the hatchery strains should be maintained, ideally, the same as that of wild populations (FAO, 1993). A population genetics approach can make fundamental contributions to achieve this purpose. There appears to be two main areas in which population genetics studies should meet the current stock enhancement concept. One is concerned with the assessment of genetic diversity and structure in the wild fish populations that should be conserved, and another is the genetic monitoring of hatchery-based fish in order to further improve the hatchery management, by which any harmful genetic impacts of stocking practice to the wild

populations can be minimized. As regards to population genetics studies of Japanese flounder, initial attempts were made by using allozyme loci as a molecular marker (Fujio *et al.*, 1985; 1989), although allozyme loci failed to detect any significant genetic structure among wild fish populations, and were insufficient to precisely evaluate the extent of the genetic variability maintained in hatchery strains due to the limited number of polymorphic loci available and the low level of polymorphisms. Researchers have therefore explored other classes of molecular markers that are suitable for Japanese flounder population genetics studies.

Microsatellite DNA loci are sequences made up of a single sequence motif such as $(CA)_n$ and $(CT)_n$, which are distributed ubiquitously throughout eukaryotic genomes (Tautz and Renz, 1984; Tautz, 1989). Microsatellites are expected to serve in a highly informative manner as a moleculer marker with the practical use in the wide range of population genetics studies (Goldstein and Schlotterer, 1999 and references therein), and as well in regard to the assessment of genetic structure as reported extensively in several fish species (*e. g.*, Rico *et al.*, 1997; Garcia de Leon *et al.*, 1997; O'Connell *et al.*, 1998; Bagley *et al.*, 1999; Chapman *et al.*, 1999; Perez-Enriquez and Taniguchi, 1999; Ruzzante *et al.*, 2000; Sekino *et al.*, 2001), genetic linkage mapping in tilapia (Kocher *et al.*, 1998), rainbow trout (Sakamoto *et al.*, 2000), Medaka (Naruse *et al.*, 2000), and Zebrafish (Shimoda *et al.*, 1999), and quantitive trait loci identification in rainbow trout (Jackson *et al.*, 1998; Sakamoto *et al.*, 1999; Danzmann *et al.*, 1999; Ozaki *et al.*, 2001). Microsatellites inhere a high amount of allelic variations with several advantages beyond other classes of molecular markers: microsatellites are amplified based on polymerase chain reaction (PCR; Saiki *et al.*, 1985) for which minute quantities and poor qualities of tissue can be used, and microsatellite analysis is comparatively easy to automate with a high throughput possible. While, the mitochondrial DNA genome (mtDNA) is predominantly inherited through a single maternal lineage with no recombination (Avise *et al.*, 1987). Theoretically, the effective population size of mtDNA is approximately one-quarter of nuclear DNA, and thus mtDNA genome seems to be susceptible to stochastic events (Carvalho and Hauser, 1994) such as population bottlenecks. The use of mtDNA as a genetic tool is therefore expected to be highly efficient to understand the genetic condition of a bottlenecked population (Kijima and Matsunami, 1992; Iguchi *et al.*, 1999). Low levels of mtDNA variability have been reported in many marine fish species (Ovenden, 1990), however, several marine fish species maintain a high amount of nucleotide variations in the mtDNA control region (*e. g.*, sword fish, Alvarado Bremer *et al.*, 1995; red sea bream, Tabata and Taniguchi, 2000). Fujii and Nishida (1997) found that the control region of Japanese flounder is also highly variable, documenting the broad applications possible to population genetics studies.

Both microsatellites and mtDNA control region offer several practical advantages in the Japanese flounder population genetics studies related to the stock enhancement. The powerful DNA techniques are expected to throw a new light on the challenging subjects such as the assessment of the genetic structure among wild fish populations or the evaluation of genetic diversity within and between hatchery strains and wild populations. The present author considers that these approaches could provide more insight into further improvement of the currently operated hatchery management.

This thesis aims to propound a view for future hatchery operations for Japanese flounder. This thesis consists of seven chapters including this introductory chapter. Chapter 2 describes the isolation and characterization of Japanese flounder microsatellites with special emphasis on an efficient cloning method of microsatellites, and the variability and inheritability of the isolated microsatellites are also examined. Chapter 3 examines the genetic diversity and structure among wild fish populations. Chapter 4 surveys several hatchery

strains evaluating the genetic diversity. Chapter 5 presents an example of parentage analysis in a hatchery strain that were created through a practical hatchery operation. Chapter 6 explores a possible way to maintain the genetic variations of captive populations in the lack of pedigree information. Chapter 7 proposes several hatchery management options to minimize the loss of genetic variations in hatchery strains, and future prospects of genetic studies in relation to Japanese flounder stock enhancement are also discussed.

Chapter 2. Isolation and characterization of Japanese flounder microsatellite DNA loci

A conventional approach to isolate microsatellite sequences has generally been used (reviewed in O'Reilly and Wright, 1995): a small-insert (300-500 base pairs) partial genomic library is constructed, and the library is screened with a repeat unit probe such as a $(CA)_n$ probe. The frequency of microsatellites in this "conventional" library is however usually low, and is expected to be of 1 per 100-400 colonies (Ostrander *et al.*, 1992), although the frequency of microsatellites varies depending on the species. The conventional method thus requires much labor, and is time consuming since a large number of colony plates need to be screened to isolate a significant number of microsatellites. This is of special concern in species with a low frequency of microsatellites in their entire genome (Takahashi *et al.*, 1996). Therefore, a method to increase the efficiency for cloning species-specific microsatellites has been required. Ostrander *et al.* (1992) proposed a method for the construction of canine genomic libraries in which microsatellites were highly enriched, and Takahashi *et al.* (1996) also developed an efficient method to isolate microsatellites from the chicken genome, of which the expected frequency of microsatellites is tenfold less than those in mammalian genomes (Crooijmans *et al.*, 1993). In this study, the method devised by Takahashi *et al.* (1996) was applied to the Japanese flounder with modifications.

Microsatellites are theoretically inherited according to Mendelian transmission, and thus the mode of microsatellite allele segregation has not been a major issue. At the same time, microsatellite analysis is conducted through the PCR amplification procedures; the misbinding of primers to complementary DNA sequences may result in scoring errors of alleles, commonly known as the presence of null alleles. This causes an apparent incompatibility of genotypes within a family when a simple Mendelian transmission of microsatellite alleles is assumed (Callen *et al.*, 1993; Ede and Crawford, 1995), or inconformity of observed genotype frequencies to Hardy-Weinberg's expectations with significant depression of observed heterozygosity from the expected one in a Mendelian population (Pemberton *et al.*, 1995). The inheritability of microsatellite loci in experimental family lineages should be thus studied prior to its application to population genetics studies.

In this chapter, the present author details the isolation technique of Japanese flounder microsatellites from the genome, and attempts were made to characterize the isolated microsatellites in terms of the variability and inheritability in order to qualify them as a valuable molecular tool.

Materials and methods

Cloning of microsatellites

Samples of muscle tissue taken from Japanese flounder were digested with Proteinase K in 4 M TNES-Urea buffer (Asahida *et al.*, 1996), and genomic DNA was purified by phenol/chloroform extraction with ethanol precipitation (Sambrook *et al.*, 1989). Genomic DNA was fragmented by sonication, and resultant fragmented DNA was blunted by mung bean nuclease (Takara, Shiga, Japan). Fragments were electrophoresed in a 1.2% agarose gel, and the fragments ranging from 300 to 500 bp were recovered. Both ends of the recovered fagments were repaired by T4 DNA polymerase (Toyobo,

Osaka, Japan) and T4 polynucleotide kinase (Toyobo). The fragments were ligated to *Srf* I site of pCR-Script Amp SK(+) phagemid vector (Stratagene, La Jolla, CA, USA), and then the recombinant phagemids were transformed into XL2-Blue MRF' competent cells (Stratagene). An equivalent to 10^{10} pfu of helper phage VCSM13 (Stratagene) was added, and the cultivation was continued overnight. After that, the phagemids were precipitated according to a standard PEG/NaCl precipitation procedure (Sambrook *et al.*, 1989). Contaminating *Escherichia coli* DNA and RNA were digested with DNase I (Takara) and RNaseA (Nacalai Tesque, Kyoto, Japan). The phagemids were recovered with PEG/NaCl precipitation, and single-stranded DNA was purified with phenol/chloroform extraction and ethanol precipitation. Selective second strand DNA synthesis *in vitro* with $(CA)_{12}$ oligonucleotide primer was carried out according to Takahashi *et al.* (1996). Single-stranded DNA remaining in the mixture was digested with mung bean nuclease (Takara). Resultant double-stranded DNA was transformed into XL2-Blue MRF' competent cells again, and the resulting transformants were referred to as a $(CA)_n$-enriched library.

From the $(CA)_n$-enriched library, individual clones were randomly picked up, and phagemid DNAs were purified using GFX *Micro* Plasmid prep kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The DNA sequences were determined in both directions using Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech) in combination with the ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech).

Microsatellite variability and inheritability

Primer pairs flanking each repeat array was developed using the Primer Premier software package (Premier Biosoft International, Palo Alto, CA, USA). Microsatellite polymorphisms were screened in a wild Japanese flounder population collected from the Japan Sea off Tottori Prefecture (69 individuals). Polymerase chain reaction (PCR) amplification was carried out in a 20 μL reaction volume, which included 20 pmols of each primer set (one primer in each of pair was 5' end-labelled with Cy5 amidite, Amersham Pharmacia Biotech), 100 μM each dNTP, 10 mM Tris-HCl (pH8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 units of DNA polymerase (AmpliTaq Gold™, Applied Biosystems, Foster City, CA, USA), and approximately 50ng of template DNA using PC-960G gradient thermal cycler (Corbett Research, Mortlake, NSW, Australia). PCR amplification cycles were as follows: 12 min at 95℃, 35-40 cycles of 30 sec at 94℃, 1 min at a primer-specific temperature, 1 min at 72℃, and final elongation for 5 min at 72℃. The specific annealing temperature of each primer set is given in Table 2. Following PCR amplification, the reaction mixture was mixed with an equal volume of loading dye (de-ionized formamide containing 0.5% blue dextran), and heated for 5-10 min at 94℃ prior to electrophoresis. Microsatellite polymorphisms were detected in polyacrylamide sequencing gels (ReproGel™ High Resolution, Amersham Pharmacia Biotech) using the ALFexpress DNA sequencer (Amersham Pharmacia Biotech). Alleles were designated according to PCR product size relative to a molecular size marker (ALFexpress Sizer™ 50-500, Amersham Pharmacia Biotech) in combination with an ALLELELINKS version 1.00 software (Amersham Pharmacia Biotech).

Allele frequencies and unbiased expected heterozygosity (H_e; Nei, 1987) at each locus were calculated using an ARLEQUIN version 1.1 software package (Schneider *et al.*, 1997). The observed heterozygosity (H_o) was calculated directly from the observed genotypes. Hardy-Weinberg equilibrium (HWE) for each locus was examined by a test analogous to the Fisher's exact test with the Markov chain procedures (Markov-chain steps, 100,000; dememorization, 10,000) by using the ARLEQUIN program.

Inheritability of the microsatellites isolated was examined using five experimental families of Japanese flounder. From five sets of simple 1×1 crosses using three ripe females (FM1,

FM2, and FM3) and four ripe males (M1, M2, M3, and M4), approximately 300 full-sibs were successfully obtained from each cross (family A: FM1×M1, family B: FM2×M1, family C: FM3×M2, family D: FM3×M3, and family E: FM3×M4). Accordingly, the members of family A and B were paternal half-sibs, and family C, D, and E consisted of maternal half-sibs. The inheritance mode of microsatellite alleles was studied in up to 30 F_1 offspring in each family. In order to determine if alleles were inherited according to simple Mendelian fashion, the conformity of the offspring genotypes to the expectations was tested using the chi-square methods (Snedecor and Cochran, 1967).

Results

Of the 80 clones randomly selected from the $(CA)_n$-enriched library, 59 clones contained one or more repeat arrays, of which 25 clones were discarded due to the unreproducible sequencing results flanking the repeat regions. Four of the remaining 34 clones were over-represented with the other clones, and consequently 33 repeat arrays were isolated from the remaining 30 clones (Table 1), of which 27 repeat arrays were available for the development of PCR primers (Table 2) and six repeat regions did not provide sufficient sequence information to develop any primer pairs.

All the 27 primer pairs were available for PCR amplification, out of which 16 primer pairs were used for further assessment of microsatellite polymorphisms in a wild Japanese flounder population. The remaining 11 primer pairs were discarded since they produced unexpected PCR products in an initial sample of Japanese flounder. As shown in Table 3, the genetic variabilities of the 16 microsatellites varied depending on the locus. The number of alleles ranged from 4-40, and the H_o and H_e values ranged from 0.43 to 0.99, and from 0.46 to 0.97, respectively. All but one of the 16 loci conformed to HWE after correction of significance levels for 16 simultaneous tests (Rice, 1989) ($p>0.003$, initial K of sequential Bonferroni correction, $K=16$); at the *Po31* locus, the observed genotype frequency showed a significant departure from HWE ($p<0.003$) with a large discrepancy between the H_o and H_e values (0.34 and 0.91, respectively).

The Mendelian inheritability of each locus was assessed by using five experimental fami-

Table 1. Core repeat sequences isolated from 30 clones randomly selected from the $(CA)_n$-enriched library

Clone No.	Repeat motif (5'-3')	Clone No.	Repeat motif (5'-3')
1	$(TG)_3T_2(TG)_8$	16	$(CACG)_4(CA)_5$
	$(CA)_2GT(AC)_{14}$	17	$(CA)_2CG(CA)_6GA(CA)_5$
2	$(TC)_7TG(TC)_9$	18	$(CAA)_2T(AC)_6(AT)_2$
	$(CA)_5A(AC)_4$	19	$(AC)_{20}$
3	$(CA)_{12}(CGCA)_2(CA)_5CG(CA)_6$	20	$(CA)_{11}(GA)_2GC(GA)_9$
4	$(TG)_3G(AC)_{13}$	21	$(CA)_4$
5	$(GAA)_3(CA)_5$	22	$(TG)_7C(GT)_5\{GC(GT)_5\}_2(CGTG)_2(TG)_4$
6	$(CACG)_4(CA)_4CG(CA)_{18}C(GT)_3$	23	$(CA)_4TA(CA)_2A_6(CA)_3$
7	$(TC)_7T2(TC)_3TA(AC)_{21}$	24	$(CA)_{48}$
8	$(AC)_4C(CA)_2G(AC)_4C(CA)_2G(AC)_5$	25	$(CA)_6T_2(CA)_{16}(CCCA)_2CA_3(CA)_2$
9	$(GATG)_2A_2CA(GATG)_{10}$	26	$(CA)_5AG(CG)_2(TG)_3(CG)_2(CA)_{15}$
	$(CA)_2GA(CA)_4GA(CA)_4$	27	$(CA)_3(AC)_2G(CA)_{26}$
10	$(CA)_6CGCACGGA(CA)_7$	28	$TA_3(CA)_7$
11	$(CA)_4(GA)_2(CA)_{11}$	29	$(CA)_{18}$
12	$(TG)_5T_2(TG)_{10}$	30	$(CACG)_2(CA)_6$
13	$(CA)_7$		
14	$(CA)_9$		
15	$(CA)_5(TA)_{13}(CA)_3$		

Table 2. Repeat motifs, PCR primer sequences, and annealing temperature of 27 Japanese flounder microsatellites

Locus	Repeat motif (5'-3')	Primer sequence (5'-3')	Annealing temperature[1] (℃)
Po 1	$(TG)_3T_2(TG)_8$	F-GCCTTTTGTCAGCCATTAACAGAGC	55
		R-CTGAGGCCAGACATGACATTACCTT	
Po7	$(CA)_5A(AC)_4$	F-AACAAACAAACCGGGCTGGCAGCG	56
		R-AGCCATGTGACGACAGACCCAGCATG	
Po9	$(CA)_{12}(CGCA)_2$	F-GCATCAGTGCGTGCAGCATGTTAGTA	58
	$(CA)_5CG(CA)_6$	R-CTGACAACAGCCGCTTTGAGCATCTA	
Po13	$(TG)_3GA(CA)_{13}$	F-CGGCCTAAACCTGGACATCCTCTCTA	58
		R-CGGGACAACGGAGGTTTGACTGAC	
Po15	$(GAA)_3(CA)_5$	F-TTCAGTACCTGTTCAACCTGATGT	52
		R-CACAACTTAAGGTAACAACCTGCT	
Po20	$(CACG)_4(CA)_4C$	F-TGCTCCTTCACCTGCACGGCCTCAAA	58
	$G(CA)_{18}C(GT)_3$	R-TGCACCCTGACCTGTCACTGGGGATT	
Po25A	$(GATG)_2A2$	F-TGAGGAGTCAGGTTTCAGGCCACT	55
	$CA(GATG)_{10}$	R-TCGCAGGAACACCCAGAGTACAGA	
Po25B	$(CA)_2GA(CA)_4$	F-AAGCTCTGTACTCTGGGTGTTCCTG	55
	$GA(CA)_4$	R-ACCAACGTCACTATCACTCCTCTGC	
Po26	$(CA)_6CGCA$	F-ACACTGGGCCCTCTGTTAAACAC	55
	$CGGA(CA)_7$	R-AGAGGAGAAAGGGCACCGAGATA	
Po31	$(CA)_4(GA)_2(CA)_{11}$	F-AGGGTTAATTATAGAGGACGCAG	57
		R-CTGAAACAACAACTCAGAAGACG	
Po33	$(TG)_5T_2(TG)_{10}$	F-GTTGGTTTAACTGATTCATCTGCAG	55
		R-TTACATATCCCACAATGCTTCACTC	
Po35	$(CA)_7$	F-TGGTTCTAGTGTTTGTCTGGTGA	54
		R-CCTACAGCACAGATATGACCTTT	
Po37	$(CA)_9$	F-ATACGACAACATCTGTCTGCAACACC	53
		R-GATAAACTTGCTGCTCCTTGTGGGTA	
Po42	$(CA)_5(TA)_{13}(CA)_3$	F-CGAGCGCTGTTTCAACTACGGTCATT	55
		R-ATGATGATCTAACCGTCCGGCTCCAT	
Po48	$(CACG)_4(CA)_5$	F-GCCTCCAGAAACATTTATGGGG	55
		R-TGTCTTGCCTCTGGTCCTTCTT	
Po52	$(CA)_2CG(CA)_6$	F-TCAGACAGAGGAGCGGGGTTGTTGC	58
	$GA(CA)_3$	R-GCTGTACCCAGGGTTCCGCTGAAGA	
Po55	$(CAA)_2T(AC)_6$	F-TCTTCTGATGCTGCCCAGGCTCCTT	58
	$(AT)_2$	R-CGACCATGATCACCCCATGAGAACG	
Po56	$(AC)_{20}$	F-TCGAGCGTAAACAAACCAGCTAACA	55
		R-GCTGAAAATCGCTTTAGCTTCCCAT	
Po58	$(CA)_{11}(GA)_2$	F-GCCCCTCACTGAGACTGTGACA	52
	$GC(GA)_9$	R-CAAGGTATGTGCATGAGCAGGC	
Po69	$(TG)_7C(GT)_5\{GC(GT)_5\}_2$	F-TGGATTGTACCTTGACTCACCTGCT	53
	$(CGTG)_2(TG)_4$	R-GTGTTAATCATATGTGCCACCAGCT	
Po74	$(CA)_4TA(CA)_2$	F-ACCTTCAGCAGCATGTCCACATAA	50
	$A_6(CA)_3$	R-CACTAGTATGCTTTGGCGGCAAAT	
Po78	$(CA)_{48}$	F-GTAGACAACAAATCGGTTGTGCATG	56
		R-CGATTGGGACATGAGTTGTTATGAG	
Po80	$(CA)_6T_2(CA)_{16}$	F-TTCTGCATAATGAATGCAGTCCTC	54
	$(CCCA)_2CA_3(CA)_2$	R-ACACGGCATGCAAATTACTACAGT	
Po83	$(CA)_5AG(CG)_2$	F-TGCGGTCATCATGTCTTTAAAATA	57
	$(TG)_3(CG)_2(CA)_{15}$	R-AGCAAATGTTTGCTTTTGGATACA	
Po89	$TA_3(CA)_7$	F-ATCAGAAGTCATCCATGCACTGGCAC	60
		R-AGCTACTTATCCACAGGTGTCGACGG	
Po91	$(CA)_{18}$	F-AGGTTTCAAGGTGTTCATTGCGAGTC	55
		R-TAAAGGAAGTGCCTCACTGTGGAGAA	
Po93	$(CACG)_2(CA)_6$	F-ACCACATTGAACGAATGACAGCG	56
		R-CTGGGACTTTCTTTGCATATCTCCTG	

lies of Japanese flounder. Of the 16 microsatellites screened, the *Po31* locus had unstable and unreproducible PCR amplification of a particular allele in one family. This locus was thus discarded, and the remaining 15 loci were used for further statistical analysis. Table 4 shows the genotype frequencies estimated for the offspring, and the parental allele segregating patterns among the offspring in the 55 possible combinations of the five families and the 15 loci. Possible scoring errors, as indicated at the *Po31* locus, were not common in the other loci, as all the expected genotypes and no unexpected ones were observed throughout all families. The genotype frequencies of offspring in each family were in accordance with the expectations for almost all of the 15 loci, excepting three instances (the *Po48* locus in family E, the *Po56* locus in family D, and the *Po58* locus in family B, $0.01 < p < 0.05$). These deviations, however, were not significant after the correction of significance levels on the basis of the sequential Bonferroni method (5 simultaneous test, initial $K=5$; significant value at the $\alpha=0.05$ was adjusted at $\alpha=0.01$; For $\alpha=0.01$, the critical χ^2 value is 9.21 for $2df$, and 13.28 for $3df$).

Discussion

Efficient method for cloning microsatellites

A schematic of the microsatellite enrichment procedures employed in this study is shown in Fig. 1. This method achieved a high efficiency in the enrichment of Japanese flounder microsatellites: approximately 75% of the clones (59/80 clones) derived from the $(CA)_n$-library contained one or more repeat arrays. Takahashi *et al.* (1996) reported that about a half of clones in the library they constructed were over-represented, and these authors speculated that the results might be caused by a severe bias of DNA fragments due to the use of restriction enzymes at the DNA-fragmentation step. The use of sonicated DNA

Table 3. Variability of the 16 microsatellite loci estimated for a wild Japanese flounder population

Locus	Sample size	No. of alleles	Size range[*1] (bp)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)	p[*2]	GenBank accession no.
Po 1	69	26	162-234	0.87	0.82	0.63	AB046745
Po13	69	27	206-254	0.97	0.92	0.99	AB046746
Po20	69	40	239-379	0.99	0.97	1.00	AB046748
Po25A	69	12	201-253	0.77	0.76	0.31	AB046749
Po26	68	5	141-159	0.74	0.65	0.69	AB046750
Po31	69	25	129-193	0.43	0.91	0.00*	AB046751
Po33	69	10	257-290	0.72	0.68	0.61	AB046752
Po35	69	19	283-333	0.81	0.78	1.00	AB046753
Po42	69	23	164-224	0.88	0.91	0.67	AB046754
Po48	64	7	126-146	0.46	0.46	0.74	AB046755
Po52	69	4	155-163	0.46	0.50	0.61	AB046756
Po56	69	26	139-205	0.94	0.94	0.77	AB046757
Po58	69	27	101-159	0.84	0.90	0.52	AB046758
Po83	68	32	227-313	0.91	0.93	0.18	AB046759
Po89	69	20	252-327	0.86	0.90	0.44	AB046760
Po91	69	34	146-246	0.96	0.94	1.00	AB046761
Mean		21.1		0.79	0.81		

[*1] Size is indicated as the number of the base pairs of PCR products

[*2] p is the exact p-value estimated by a test anologous to Fisher's exact test described by Schneider *et al.* (1997). Significant departure of the observed genotype frequencies from HW-expectations for each locus was determined by adding $*=p<0.003$ with initial K of sequential Bonferroni correction (Rice, 1989), K=16.

Table 4. Inheritance characteristics of microsatellite alleles in 5 experimental families of Japanese flounder

Locus	Family	Parental genotypes	N*1	Observed number of offspring in each genotypic class*2				χ^2
Po1	A	♂162/180		162/162	162/172	162/180	172/180	
		♀162/172	30	11(7.5)	5(7.5)	7(7.5)	7(7.5)	2.53
	B	♂162/180		162/164	162/214	164/180	180/214	
		♀164/214	30	7(7.5)	5(7.5)	10(7.5)	8(7.5)	1.73
	C	♂162/162		162/162	162/214			
		♀162/214	30	16(15.0)	14(15.0)			0.13
	D	♂162/162		162/162	162/214			
		♀162/214	30	16(15.0)	14(15.0)			0.13
	E	♂162/162		162/162	162/214			
		♀162/214	30	15(15.0)	15(15.0)			0.00
Po13	A	♂216/230		216/216	216/230			
		♀216/216	30	14(15.0)	16(15.0)			0.13
	B	♂216/230		212/216	212/230	216/268	230/268	
		♀212/268	30	11(7.5)	8(7.5)	5(7.5)	6(7.5)	2.53
	C	♂214/228		214/214	214/216	214/228	216/228	
		♀214/216	30	5(7.5)	8(7.5)	12(7.5)	5(7.5)	4.40
	D	♂208/240		208/214	208/216	214/240	216/240	
		♀214/216	30	12(7.5)	3(7.5)	8(7.5)	7(7.5)	5.47
	E	♂214/246		214/214	214/216	214/246	216/246	
		♀214/216	30	8(7.5)	9(7.5)	8(7.5)	5(7.5)	1.20
Po20	A	♂253/255		253/259	255/259			
		♀259/259	30	11(15.0)	19(15.0)			2.13
	B	♂253/255		239/253	239/255	253/263	255/263	
		♀239/263	29	6(7.25)	11(7.25)	3(7.25)	9(7.25)	5.07
	C	♂237/307		237/263	237/293	263/307	293/307	
		♀263/293	30	9(7.5)	6(7.5)	10(7.5)	5(7.5)	2.27
	D	♂265/307		263/265	263/307	265/293	293/307	
		♀263/293	30	7(7.5)	11(7.5)	10(7.5)	5(7.5)	3.33
	E	♂263/271		263/263	263/271	263/293	271/293	
		♀263/293	30	6(7.5)	7(7.5)	9(7.5)	8(7.5)	0.67
Po25A	A	♂229/253		221/229	221/253	225/229	225/253	
		♀221/225	30	4(7.5)	5(7.5)	12(7.5)	9(7.5)	5.47
	B	♂229/253		221/229	221/253	229/229	229/253	
		♀221/229	30	7(7.5)	9(7.5)	10(7.5)	4(7.5)	2.80
	C	♂225/229		225/225	225/229			
		♀225/225	30	18(15.0)	12(15.0)			1.20
	D	♂225/229		225/225	225/229			
		♀225/225	30	19(15.0)	11(15.0)			2.13
	E	♂225/225		225/225				
		♀225/225	30	30(30.0)				—
Po26	A	♂141/159		141/155	155/159			
		♀155/155	30	18(15.0)	12(15.0)			1.20
	B	♂141/159		141/155	141/157	155/159	157/159	
		♀155/157	30	10(7.5)	5(7.5)	3(7.5)	12(7.5)	3.53
	C	♂141/155		141/141	141/155	155/155		
		♀141/155	30	10(7.5)	15(10.0)	5(7.5)		4.17
	D	♂155/155		141/155	155/155			
		♀141/155	30	19(15.0)	11(15.0)			2.13
	E	♂141/155		141/141	141/155	155/155		
		♀141/155	30	5(7.5)	20(15.0)	5(7.5)		3.34
Po33	A	♂263/263		263/263	263/286			
		♀263/286	30	14(15.0)	16(15.0)			0.13
	B	♂263/263		263/263	263/288			
		♀263/288	30	13(15.0)	17(15.0)			0.53
	C	♂263/265		263/263	263/265			
		♀263/263	30	12(15.0)	18(15.0)			1.20
	D	♂265/286		263/265	263/286			
		♀263/263	30	13(15.0)	17(15.0)			0.53
	E	♂263/265		263/263	263/265			
		♀263/263	30	17(15.0)	13(15.0)			0.53
Po35	A	♂285/317		285/285	285/317			
		♀285/285	30	17(15.0)	13(15.0)			0.53
	B	♂285/317		285/285	285/317	317/317		
		♀285/317	30	9(7.5)	14(15.0)	7(7.5)		0.40
	C	♂285/291		285/285	285/291			
		♀285/285	30	15(15.0)	15(15.0)			0.00
	D	♂285/317		285/285	285/317			
		♀285/285	30	15(15.0)	15(15.0)			0.00
	E	♂285/289		285/285	285/289			
		♀285/285	30	16(15.0)	14(15.0)			0.13
Po42	A	♂176/206		176/180	176/200	180/206	200/206	
		♀180/200	30	7(7.5)	10(7.5)	8(7.5)	5(7.5)	1.73
	B	♂176/206		172/176	172/206	176/180	180/206	
		♀172/180	30	4(7.5)	13(7.5)	7(7.5)	6(7.5)	6.00
	C	♂182/200		182/200	200/200			
		♀200/200	30	18(15.0)	12(15.0)			1.20
	D	♂198/198		198/200				
		♀200/200	30	30(30.0)				—
	E	♂176/178		176/200	178/200			
		♀200/200	30	16(15.0)	14(15.0)			0.13

*1 N is the number of offspring analyzed

*2 Mendelian expectations in each genotypic class are shown in parentheses. Significant deviation of the observed genotype frequencies from the expectations was determined by adding $*p<0.05$. The critical χ^2 value is: 7.82 for 3*df*, 5.99 for 2df, 3.84 for 1*df*. In 3 stances (the *Po52* locus in family A, the *Po42* locus in family D, and the *Po25A* locus in family E), chi-square analysis was not performed since both parents possessed homozygous genotypes.

Table 4. (continued)

Locus	Family	Parental genotypes	N[1]	Observed number of offspring in each genotypic class[2]				χ^2
Po48	A	♂126/130		126/126	126/130			
		♀126/126	30	16(15.0)	14(15.0)			0.13
	B	♂126/130		126/126	126/130			
		♀126/126	30	15(15.0)	15(15.0)			0.00
	C	♂126/126		126/126	126/130			
		♀126/130	30	15(15.0)	15(15.0)			0.00
	D	♂126/130		126/126	126/130	130/130		
		♀126/130	30	5(7.5)	16(15.0)	9(7.5)		1.20
	E	♂126/126		126/126	126/130			
		♀126/130	29	9(14.5)	20(14.5)			4.17*
Po52	A	♂155/155		155/155				
		♀155/155	30	30(30.0)				—
	B	♂155/155		155/155	155/161			
		♀155/161	30	15(15.0)	15(15.0)			0.00
	C	♂155/161		155/161	155/163	161/161	161/163	
		♀161/163	30	6(7.5)	6(7.5)	12(7.5)	6(7.5)	3.60
	D	♂161/161		161/161	161/163			
		♀161/163	30	16(15.0)	14(15.0)			0.13
	E	♂155/155		155/161	155/163			
		♀161/163	30	18(15.0)	12(15.0)			1.20
Po56	A	♂159/177		159/175	175/177			
		♀175/175	30	15(15.0)	15(15.0)			0.00
	B	♂159/177		159/163	159/173	163/177	173/177	
		♀163/173	30	4(7.5)	7(7.5)	7(7.5)	12(7.5)	4.40
	C	♂159/165		159/163	159/173	163/165	165/173	
		♀163/173	30	8(7.5)	5(7.5)	5(7.5)	12(7.5)	4.40
	D	♂161/165		161/163	161/173	163/165	165/173	
		♀163/173	30	4(7.5)	14(7.5)	6(7.5)	6(7.5)	7.87*
	E	♂171/177		163/171	163/177	171/173	173/177	
		♀163/173	30	4(7.5)	10(7.5)	8(7.5)	8(7.5)	2.53
Po58	A	♂115/117		111/115	111/117	115/153	117/153	
		♀111/153	28	9(7.0)	3(7.0)	7(7.0)	9(7.0)	3.43
	B	♂115/117		115/117	115/139	117/117	117/139	
		♀117/139	30	7(7.5)	15(7.5)	5(7.5)	3(7.5)	11.27*
	C	♂117/123		115/117	115/123	117/117	117/123	
		♀115/117	30	4(7.5)	9(7.5)	4(7.5)	13(7.5)	7.60
	D	♂111/147		111/115	111/117	115/147	117/147	
		♀115/117	30	6(7.5)	9(7.5)	10(7.5)	5(7.5)	2.27
	E	♂125/125		115/125	117/125			
		♀115/117	30	14(15.0)	16(15.0)			0.13

Locus	Family	Parental genotypes	N	Observed number of offspring in each genotypic class				χ^2
Po83	A	♂275/287		253/275	253/287	275/301	287/301	
		♀253/301	30	5(7.5)	7(7.5)	7(7.5)	11(7.5)	2.53
	B	♂275/287		271/275	271/287			
		♀271/271	30	15(15.0)	15(15.0)			0.00
	C	♂233/271		233/273	233/275	271/273	271/275	
		♀273/275	30	7(7.5)	5(7.5)	11(7.5)	7(7.5)	2.53
	D	♂261/271		261/273	261/275	271/273	271/275	
		♀273/275	30	7(7.5)	6(7.5)	13(7.5)	4(7.5)	6.00
	E	♂273/273		273/273	273/275			
		♀273/275	20	11(10.0)	9(10.0)			0.20
Po89	A	♂283/295		268/283	268/295	270/283	270/295	
		♀268/270	29	10(7.25)	6(7.25)	3(7.25)	10(7.25)	4.79
	B	♂283/295		274/283	274/295	279/283	279/295	
		♀274/279	30	9(7.5)	10(7.5)	3(7.5)	8(7.5)	3.87
	C	♂268/270		268/268	268/270	268/279	270/279	
		♀268/279	30	10(7.5)	8(7.5)	7(7.5)	5(7.5)	1.73
	D	♂268/270		268/268	268/270	268/279	270/279	
		♀268/279	30	8(7.5)	8(7.5)	8(7.5)	6(7.5)	0.40
	E	♂270/279		268/270	268/279	270/279	279/279	
		♀268/279	30	7(7.5)	8(7.5)	8(7.5)	7(7.5)	0.13
Po91	A	♂146/162		146/148	146/188	148/162	162/188	
		♀148/188	30	10(7.5)	7(7.5)	7(7.5)	6(7.5)	0.93
	B	♂146/162		146/158	146/160	158/162	160/162	
		♀158/160	29	7(7.25)	7(7.25)	8(7.25)	7(7.25)	0.10
	C	♂192/228		148/192	148/228	160/192	160/228	
		♀148/160	30	5(7.5)	6(7.5)	9(7.5)	10(7.5)	2.27
	D	♂158/192		148/158	148/192	158/160	160/192	
		♀148/160	29	9(7.25)	9(7.25)	6(7.25)	5(7.25)	1.76
	E	♂150/162		148/150	148/162	150/160	160/162	
		♀148/160	30	8(7.5)	7(7.5)	5(7.5)	10(7.5)	1.73

fragments is a possible improvement in this regard as approximately 90% of the clones sequenced were unique (30/34 clones). Likewise, Sekino and Hara (2001) reported that this enrichment procedure was also efficient to clone the Pacific abalone *Haliotis discus* microsatellites: they succeeded in the construction of overall 400-fold enriched library than that constructed based on the conventional method, and all the 39 clones for which they sequenced were unique.

Microsatellite variability and inheritability

The high level of genetic variability detected in the 16 microsatellites suggests that these loci provide greater amounts of genetic information compared with those of the allozymic loci reported (Fujio *et al.*, 1989): four wild populations of Japanese flounder screened for 23 enzyme loci revealed that the average number of alleles

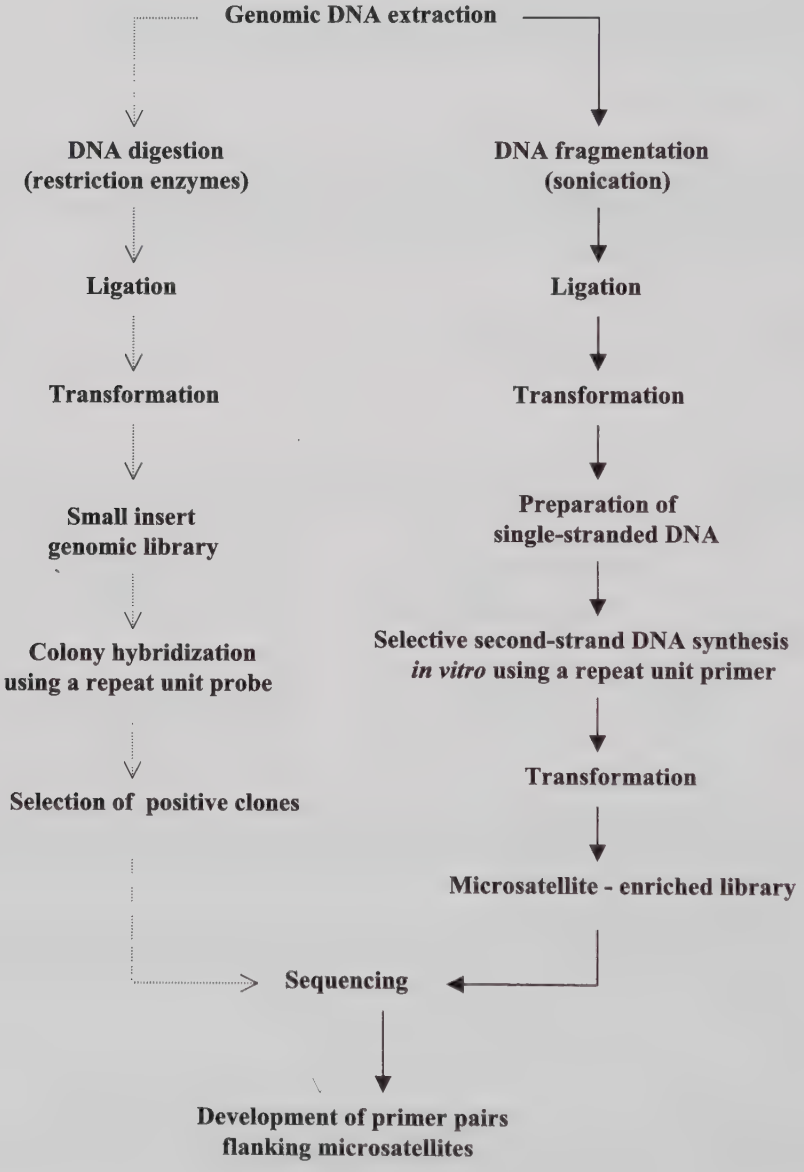

Fig. 1. A schema of the procedures for cloning microsatellites. Dotted lines indicate the conventional method described by Brooker *et al.* (1994), and thick lines indicate the enrichment procedures described by Takahashi *et al.* (1996) and Sekino *et al.* (2000).

per locus (A) and the average of the H_e value were 1.7 and 0.04, respectively. The A and mean H_e values estimated for the Japanese flounder microsatellites (A: 21.1, H_e: 0.81) were roughly similar to those reported in other marine fish microsatellites; as an average of 66 loci in 12 species surveyed, the A and H_e values were 19.9±6.6 and 0.77±0.19 (mean±s. d.), respectively (DeWoody and Avise, 2000). A significant departure from HWE was observed at the *Po31* locus with a large discrepancy between the H_o and H_e value (0.34 and 0.91, respectively). This may be explained by sampling errors due to the limited sample size or substructuring of the samples, however, these seem unlikely since the observed genotype frequencies for all the other loci were consistent with the HWE expectations. Given the fact that the Mendelian inheritability of this locus was not verified in a particular family, the presence of null alleles possibly caused by mutations occurred within the primer binding sites (Callen *et al.*, 1993; Ede and Crawford, 1995; Pemberton *et al.*, 1995) would be most likely to cause these results. Except for the *Po31* locus, it can be concluded that the remaining 15 loci were segregated according to Mendelian transmission.

Summary

This chapter can be summarized by the following two points. First, a highly microsatellite enriched library of Japanese flounder was constructed. Given that the efficient method was applicable to the Pacific abalone (Sekino and Hara, 2001) and the Japanese rockfish *Sebastes thompsoni* (Sekino *et al.*, 2000), it is expected to be generally applicable to other marine organisms. Secondly, the microsatellite markers developed in this study, which possess hyper-variability and Mendelian inheritability, proved to be useful in further applications to various genetic studies of Japanese flounder.

Chapter 3. Genetic diversity and structure of wild Japanese flounder populations

Long distance movements related to feeding or reproduction by adult Japanese flounder are thought to be relatively limited because of their sedentary behavior (reviewed in Minami, 1997), and thus the wide range of distributions seems largely to be related to the extent of egg and larval transport at the pelagic stages that last for as long as 25-50 days (Ochiai and Tanaka, 1986). While geographical variations in morphological and biological traits such as dorsal and anal ray counts of larvae (Kinoshita *et al.*, 2000), growth rate, and fecundity (reviewed in Minami, 1997) have been reported. Although these findings possibly indicate that this species possesses locally adapted populations, little is known about the population genetic structure. Attempts from genetic perspectives have been made to understand as much about the baseline genetic structure by using two classes of molecular markers, that is, allozyme analyses (Fujio *et al.*, 1985; Fujio *et al.*, 1989) and PCR-RFLP mitochondrial DNA (mtDNA) analysis (Asahida *et al.*, 1998): however any significant genetic heterogeneities between populations have not been conclusively demonstrated. These genetic studies indicate that Japanese flounder populations around Japan may have an overall genetic homogeneity. As discussed for other marine fish populations (reviewed in Carvalho and Hauser, 1994), the lack of significant population genetic heterogeneities may be due to an interpopulational homogenization of alleles (or haplotypes) resulting from temporally and/or spatially wide-ranging gene flow among population components, which may be related to the expansive nature of marine environments and the inherent role of the pelagic stages in their life cycles. Indeed, three major oceanic currents flowing around Japan seem to be effective to transport eggs and larvae over long distances. The Tsushima Warm Current flows northeastward from the Japanese coast side of the East China Sea through the Japan Sea, while the Kuroshio Warm Current flows northeastward from the southwestern part of the Japanese coast side of the Pacific Ocean. The Oyashio

Cold Current, which flows southward from the northern part of the Japanese coast side of the Pacific Ocean, encounters the Kuroshio Warm Current at the central part of the Japanese coast side of the Pacific Ocean. In the Japan Sea, it appears that pelagic larvae can be carried approximately 660km by the Tsushima Warm Current (Kinoshita *et al.*, 2000). It is therefore probable to consider that larval passive dispersions by efficient means of oceanic currents may act to homogenize genetic compositions of Japanese flounder around Japan, even if the extent of adult movements is limited. However, other possibilities should be taken into consideration in that the population homogeneities revealed in previous genetic studies may be only due to insufficient information content of the genetic markers employed. A finding of population heterogeneities by means of the sequencing analysis of mtDNA control region (Fujii and Nishida, 1997) seems to indicate that the other classes of highly polymorphic markers may provide a clearer measure of genetic structure in this species.

This chapter assesses whether the wild Japanese flounder populations around Japan should be considered as a single panmictic population or not, and an attempt is made to provide an insight into the genetic relationships among wild populations on a large oceanographic scale basis. Eleven of the 15 microsatellite markers described in the preceding chapter coupled with a section of mtDNA control region were used for these purposes.

Materials and methods

Fish samples

A total of 401 individuals of fish samples were collected at seven sites around Japan including three populations from the Japanese coast side of the Japan Sea in Hokkaido, Niigata, and Tottori Prefectures; three populations from the Japanese coast side of the Pacific Ocean in Iwate, Chiba, and Hyogo Prefectures; and one population from the Japanese coast side of the East China Sea in Nagasaki Prefecture. These fish samples were collected leaving out any individuals who showed hypermelanosis on the blind body side so as to minimize the possible contamination by released hatchery fish: almost all the hatchery-based flounder show blind-side pigmentation, and this provides an efficient indicator to discriminate wild fish from hatchery fish (Seikai, 1997). The localities where each population sample was collected, population abbreviations, dates of sampling, and numbers of individuals sampled are given in Table 5, and the geographical positions of the populations are plotted in Fig. 2.

Microsatellite analysis

PCR primers for the 11 microsatellites described in the preceding chapter (*Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48, Po52, Po56*, and *Po91*) were used to amplify each locus from each flounder DNA sample. PCR amplification for each locus was carried out in the same manner described in the preceding chapter.

Allele frequencies and gene diversity (unbiased expected heterozygosity: H_e) in each population at each locus were calculated using the ARLEQUIN version 1.1 software package (Schneider *et al.*, 1997). The observed heterozygosity (H_o) was calculated directly from observed genotypes. Effective number of alleles (a_e) was estimated based on the formula: $a_e = 1/\Sigma x_i^2$, where x_i is the frequency of the i th allele for each locus (Crow and Kimura, 1965). An estimate of the effective population size under the infinite allele model (IAM; Kimura and Crow, 1964) was calculated according to the formula: $N_e = (H_e/(1\text{-}H_e))/4\mu$, where μ represents the mutation rate per gamete and per generation at microsatellite loci (Crow and Kimura, 1970). In this study, the average microsatellite mutation rate was assumed as of 10^{-4} following Bagley *et al.* (1999) and Garcia de Leon *et al.* (1997) since microsatellite mutation rates are likely to be in the order of between 10^{-5} and 10^{-3} (Dallas, 1992; Weber and Wong, 1993; Ellegren, 1995), and since there have been

limited data available regarding the mutation rate for the 11 microsatellite loci.

Hardy-Weinberg equilibrium (HWE) for each population at each locus was addressed using a test analogous to the Fisher's exact test in the Markov chain procedures (Markov-chain steps, 100,000; dememorization, 10,000). A fixation index (F_{IS} : Weir and Cockerham, 1984) by which HWE departures within a population can be measured was also estimated evaluating the

Table 5. Sites employed in population analysis of wild Japanese flounder

Locality	Abbreviation	Date sampled	Sample size
Hokkaido	HKD	May, 2000	50
Niigata	NGT	Dec., 1998	50
Tottori	TTR	Nov., 1998	69
Nagasaki	NSK	Jul., 1999	71
Iwate	IWT	Sep., 1997	45
Chiba	CHB	Jan., 1999	72
Hyogo	HYG	Jan., 2000	44

For sampling localities see Fig. 2

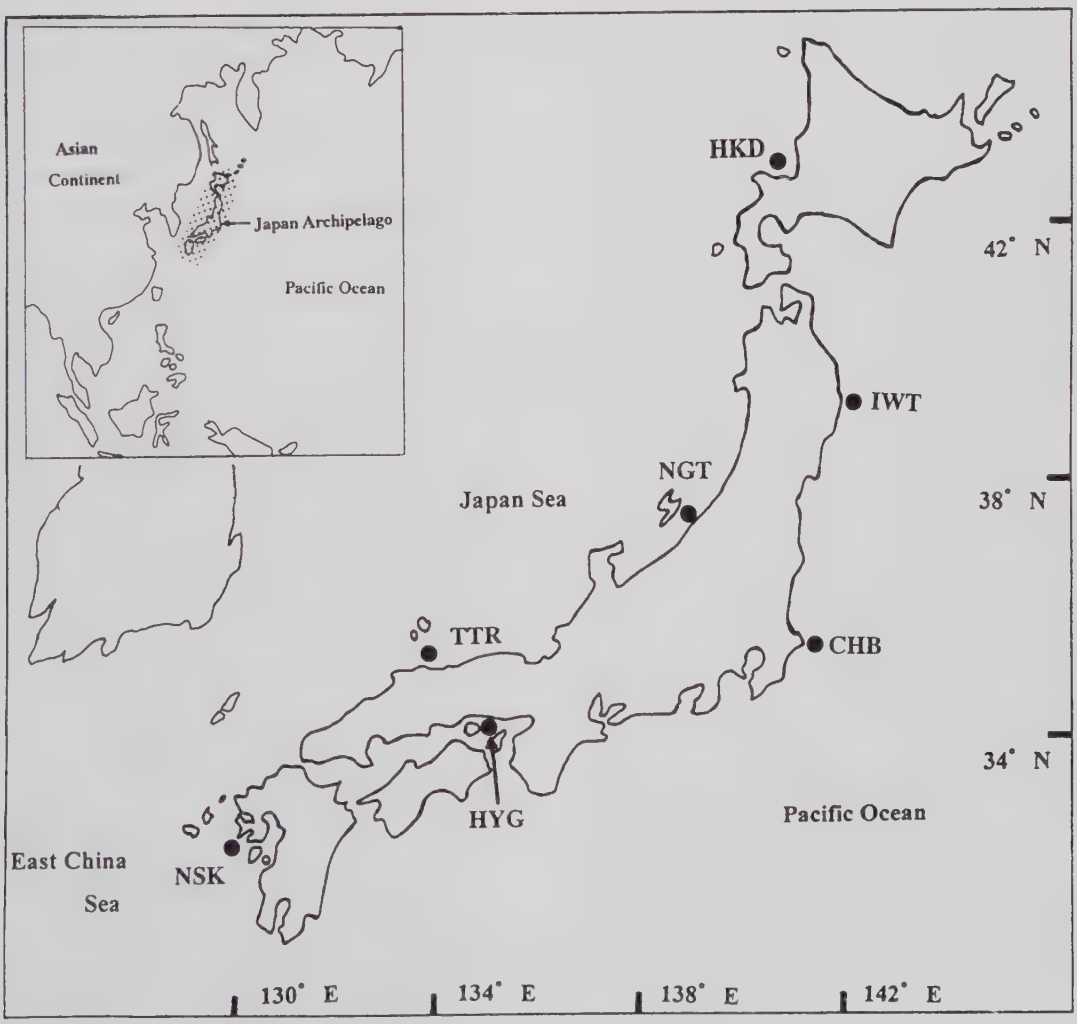

Fig. 2. A map showing sampling locations and abbreviated names for 7 Japanese flounder populations collected from the coastal sea around Japan

significance through random allelic permutation procedures (minimum 10,000 permutations). Genetic structure between populations was assessed by means of comparison of allele frequency distributions together with the tests of the significance of pairwise F_{ST} values (Reynolds *et al.*, 1983; Slatkin, 1995) for all possible population combinations. The probability associated with allele frequency homogeneity was evaluated by a test analogous to the Fisher's exact test using the Markov-chain method. The significance of pairwise F_{ST} values was evaluated through random allelic permutation procedures. These statistical analyses were conducted using the ARLEQUIN program. Significance values for all multiple tests were adjusted following the sequential Bonferroni procedures (Rice, 1989).

Genetic population relationships were estimated constructing neighbor-joining trees (NJ tree) according to the neighbor-joining method (Saitou and Nei, 1987) based on the modified Cavalli-Sforza chord distance (D_A ; Nei *et al.*, 1983) and Nei's standard genetic distance (D_{ST} ; Nei, 1972). The two distance measures were chosen to estimate population relationships since Takezaki and Nei (1996) revealed that D_A is one of the most efficient distance measures in tree topology reconstruction, whereas D_{ST} is more suitable than other distance measures in branch length estimation. The bootstrap values for each tree were calculated by 1,000 bootstrap re-sampling across loci. These procedures were performed on the NJBAFD program (provided by Dr. N. Takezaki, National Institute of Genetics, Mishima, Shizuoka, Japan), and the trees were visualized using the TREEVIEW program (Page, 1996).

A hierarchical Analysis of Molecular Variance (AMOVA: Excoffier *et al.*, 1992) was performed using the ARLEQUIN program to assess whether the groupings of populations estimated from the NJ tree topologies were supported by this approach or not. If the fixation index over all loci (Weir and Cockerham, 1984) among populations within a group (F_{SC}) significantly departed from zero, populations in the group were possibly considered to be subdivided further. Significance associated with the fixation index was evaluated thorough random allelic permutation procedures (minimum 10,000 permutations).

Mitochondrial DNA sequencing analysis

According to the complete nucleotide sequence of Japanese flounder mtDNA genome (Saitoh *et al.*, 2000; GenBank accession AB028664), one set of PCR primer pair was designed to amplify approximately 480 base pairs (bp) segment including the $tRNA^{Pro}$ gene and the left domain of the control region: two primers were placed in the $tRNA^{Thr}$ gene (forward primer: 5'-GTT AGA GCG CCA GTC TTG TA-3') and the middle of the control region (reverse primer: 5'-CCT GAA GTA GGA ACC AAA TGC-3'). PCR amplification was carried out in a 10 μL reaction mixture, which included 10 pmols of each primer, 100 μM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2 units of DNA polymerase (ExTaq™, Takara, Shiga, Japan), and approximately 50 ng of template DNA. PCR cycles were as follows: 3 min at 95℃, 30 cycles of 15 sec at 95℃, 30 sec at 57℃, 30 sec at 72℃, and final elongation for 5 min at 72℃. Following the PCR amplification, any unconsumed dNTPs and primers were digested with exonuclease I together with shrimp alkaline phosphatase (ExoSAP-IT kit, USB, Cleveland, Ohio). Sequencing analysis for the PCR amplification products was performed using the ABI 373A stretch DNA sequencer (Applied Biosystems) in combination with a Thermo Sequenase™ II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech). Sequences were determined from both directions.

Sequence alignment of mtDNA sequence data was performed by hand using the sequence editor DNASIS software package (Hitach, Tokyo, Japan). The number of variable sites, haplotype frequency distributions, haplotype diversity (h: Nei and Tajima, 1981), and nucleotide diversity (π : Nei, 1987) were calculated using the ARLEQUIN program. This software program

was also used to estimate pairwise Φ_{ST} distance, which is an analogous measure of the F_{ST} distance (Weir and Cockerham, 1984), to measure the level of population differentiation. Probability associated with the Φ_{ST} values was evaluated through random permutation procedures (minimum 10,000 permutations). A hierarchical AMOVA test was performed using the ARLEQUIN program to assess whether the grouping of populations estimated from the microsatellite data was supported by the mtDNA sequence data.

Results

Microsatellite Variability

Table 6 summarizes the genetic variability estimated for each population at each locus (allele frequency distributions for all populations and loci, see Appendix 1). One individual derived from the IWT population showed very weak PCR amplifications for all loci, and thus this individual was excluded from the data. All loci showed polymorphisms in all populations: the allelic diversity (numbers of alleles per locus: A) in the seven populations ranged from 15.2 to 18.2, while the effective number of alleles ranged from 7.2 to 9.2, and the average of the observed heterozygosity (H_o) and gene diversity (H_e) ranged from 0.74 to 0.78, and from 0.74 to 0.76, respectively. There did not appear to be large differences in the genetic variabilities among the seven populations. Four loci, *Po13, Po42, Po56*, and *Po91* harbored a very high level of allelic variation: 20 or more alleles were detected, and the H_e values were more than 0.90 in all populations. There was little discrepancy between the H_o and H_e values in each population at each locus excepting the *Po42* locus in the IWT population (H_o: 0.80 and H_e: 0.91), and consequently the H_o/H_e values on average ranged from 1.00 to 1.03 across the seven populations. Just one instance showed a significant HWE departure (at the *Po42* locus in the IWT population) after correction of significance levels for 11 simultaneous tests ($p<0.005$). The F_{IS} value estimated for the IWT population at the *Po42* locus was also significantly different from zero ($p<0.005$). The estimates of the effective population size (N_e) ranged from 7,100-7,900 with an average of 7,600 among the seven populations (Table 6).

Mitochondrial DNA sequence variability

Approximately 443 bp sequences containing the $tRNA^{Pro}$ gene (71 bp) and the left domain of the control region of mtDNA for a total of 399 individuals were unambiguously determined. It was unable to determine the sequences of one individual from each of the CHB and NSK populations due probably to the poor quality of the template DNA. As shown in Fig. 3 and Table 7, the mtDNA control region of Japanese flounder was highly variable (see also Fujii and Nishida, 1997), and all but three of the variable sites were contained in the control region. There were 150 variable sites comprised of 170 base-substitutions (138 transitions and 32 transversions) with seven indels (single base pair insertion/deletion). At one sequence site (sequence No. 139), almost all the variations detected were transversions. Eleven variable sites (sequence No. 139, 153, 183, 219, 220, 225, 259, 327, 385, 398, and 430) showed especially high levels of polymorphisms. Table 8 summarizes the sequence and haplotype variabilities estimated for each of the seven populations. A total of 287 haplotypes were identified among 399 individuals. The number of observed haplotypes in the seven populations varied from 39 to 65, and more than 50 % of the haplotypes identified in each population were unique. It appears that the number of haplotypes commonly shared between populations and the geographical locations of populations were not correlative (Table 10); for example, the CHB and IWT populations located on the Pacific Ocean commonly shared nine and 10 haplotypes with the TTR population located on the Japan Sea, respectively, while the CHB and IWT population had just three common haplotypes in spite of their geographically proximal locations. The estimates of nucleotide diversity (π) ranged from 0.027 to 0.032, and the difference of the

Table 6. Number of alleles (A), effective number of alleles[*1] (a_e), observed heterozygosity (H_o), expected heterozygosity (H_e), F_{IS} values[*2] and effective population size[*3] (N_e) estimated for the 7 wild Japanese flounder populations on the basis of the 11 microsatellite loci

Locus		Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po1	N	50	50	69	71	44	72	44
	$A(a_e)$	24(7.7)	22(7.7)	26(5.6)	22(5.0)	22(6.3)	28(5.3)	22(5.3)
	H_o	0.86	0.88	0.87	0.76	0.86	0.81	0.82
	H_e	0.87	0.87	0.82	0.80	0.84	0.81	0.81
	p[*4]	0.35	0.24	0.63	0.11	0.48	0.37	0.91
	F_{IS}	0.006(0.35)	-0.009(0.46)	-0.064(0.96)	0.048(0.18)	-0.035(0.65)	0.016(0.42)	-0.014(0.68)
Po13	N	50	50	69	71	44	72	44
	$A(a_e)$	25(16.7)	23(12.5)	27(12.5)	26(16.7)	25(16.7)	26(16.7)	21(11.1)
	H_o	0.90	0.84	0.97	0.92	0.91	0.97	0.93
	H_e	0.94	0.92	0.92	0.94	0.94	0.94	0.91
	p	0.07	0.09	0.99	0.52	0.80	0.09	0.95
	F_{IS}	0.041(0.19)	0.088(0.02)	-0.053(0.98)	0.023(0.28)	0.035(0.15)	-0.038(0.95)	-0.022(0.57)
Po25A	N	50	49	69	71	44	71	44
	$A(a_e)$	11(5.9)	13(5.6)	12(4.2)	13(5.6)	12(5.0)	15(5.6)	13(6.7)
	H_o	0.74	0.88	0.77	0.86	0.77	0.79	0.93
	H_e	0.83	0.82	0.76	0.82	0.80	0.82	0.85
	p	0.05	0.57	0.31	0.86	0.61	0.36	0.75
	F_{IS}	0.113(0.03)	-0.078(0.93)	-0.012(0.63)	-0.031(0.76)	0.038(0.35)	0.037(0.19)	-0.068(0.81)
Po26	N	50	50	68	71	44	72	43
	$A(a_e)$	6(2.6)	4(2.2)	5(2.9)	5(2.8)	5(3.0)	5(3.0)	5(2.6)
	H_o	0.68	0.52	0.74	0.70	0.77	0.67	0.70
	H_e	0.62	0.55	0.65	0.64	0.67	0.67	0.62
	p	0.90	0.64	0.69	0.95	0.16	0.74	0.15
	F_{IS}	-0.092(0.86)	0.057(0.23)	-0.131(0.96)	0.079(0.87)	-0.151(0.96)	0.009(0.51)	-0.057(0.76)
Po33	N	50	49	69	71	44	72	44
	$A(a_e)$	7(2.5)	7(2.6)	10(3.1)	11(3.1)	9(2.8)	9(2.7)	8(2.3)
	H_o	0.68	0.59	0.72	0.76	0.70	0.58	0.48
	H_e	0.60	0.61	0.68	0.68	0.64	0.63	0.57
	p	0.47	0.20	0.61	0.36	0.97	0.02	0.39
	F_{IS}	-0.141(0.97)	0.068(0.26)	-0.065(0.89)	-0.098(0.95)	-0.109(0.92)	0.076(0.17)	0.165(0.11)
Po35	N	50	50	69	71	44	71	44
	$A(a_e)$	17(5.0)	18(5.0)	19(4.5)	19(5.0)	16(5.3)	18(5.3)	14(4.2)
	H_o	0.84	0.76	0.81	0.80	0.82	0.81	0.70
	H_e	0.80	0.80	0.78	0.80	0.81	0.81	0.76
	p	0.45	0.08	1.00	0.72	0.97	0.14	0.08
	F_{IS}	-0.051(0.73)	0.056(0.21)	-0.046(0.86)	-0.002(0.57)	-0.005(0.61)	-0.007(0.62)	0.071(0.20)
Po42	N	50	50	69	71	44	71	44
	$A(a_e)$	23(10.0)	27(11.1)	23(11.1)	24(11.1)	25(16.7)	28(14.3)	22(10.0)
	H_o	0.96	0.94	0.88	0.90	0.80	0.93	0.95
	H_e	0.90	0.91	0.91	0.91	0.94	0.93	0.90
	p	0.87	0.58	0.64	0.50	0.000*	0.33	0.33
	F_{IS}	-0.067(0.98)	-0.031(0.68)	0.025(0.18)	0.010(0.45)	0.159*(0.001)	0.000(0.57)	-0.056(0.95)
Po48	N	50	50	64	71	44	72	44
	$A(a_e)$	6(2.0)	7(1.7)	7(1.9)	8(1.7)	6(1.7)	9(1.9)	6(2.0)
	H_o	0.54	0.42	0.46	0.46	0.39	0.47	0.43
	H_e	0.50	0.42	0.46	0.42	0.41	0.48	0.51
	p	0.49	0.87	0.74	0.22	0.89	0.34	0.16
	F_{IS}	-0.072(0.66)	0.006(0.55)	-0.006(0.58)	-0.110(0.92)	0.063(0.37)	0.023(0.45)	0.156(0.12)
Po52	N	50	50	69	70	44	72	44
	$A(a_e)$	4(1.5)	4(1.7)	4(2.0)	4(2.0)	6(1.6)	4(1.5)	4(1.9)
	H_o	0.34	0.38	0.46	0.51	0.41	0.33	0.41
	H_e	0.34	0.40	0.50	0.51	0.39	0.32	0.46
	p	0.88	0.13	0.61	0.26	0.06	0.66	0.07
	F_{IS}	-0.051(0.54)	0.040(0.26)	0.076(0.25)	-0.018(0.63)	-0.063(0.80)	-0.054(0.81)	0.102(0.23)
Po56	N	50	50	69	71	44	71	44
	$A(a_e)$	22(16.7)	22(16.7)	26(16.7)	24(14.3)	28(25.0)	23(14.3)	22(16.7)
	H_o	0.96	0.94	0.94	0.86	1.00	0.93	0.95
	H_e	0.94	0.94	0.94	0.93	0.96	0.93	0.94
	p	0.49	0.11	0.77	0.29	1.00	0.95	0.16
	F_{IS}	-0.024(0.62)	-0.002(0.38)	0.002(0.54)	0.064(0.03)	-0.048(1.00)	0.000(0.57)	-0.021(0.79)
Po91	N	50	50	69	71	44	72	44
	$A(a_e)$	23(14.3)	26(12.5)	34(16.7)	30(20.0)	28(16.7)	35(20.0)	30(20.0)
	H_o	0.94	0.94	0.96	0.97	0.98	0.96	1.00
	H_e	0.93	0.92	0.94	0.95	0.95	0.95	0.95
	p	0.98	0.67	1.00	0.46	0.98	1.00	0.41
	F_{IS}	-0.011(0.48)	-0.017(0.54)	-0.015(0.77)	-0.028(0.76)	-0.031(0.91)	-0.012(0.74)	-0.050(1.00)
Mean	$A(a_e)$	15.3(7.7)	15.7(7.2)	17.5(7.4)	16.9(7.9)	16.5(9.2)	18.2(8.2)	15.2(7.5)
	H_o	0.77	0.74	0.78	0.77	0.76	0.75	0.75
	H_e	0.75	0.74	0.76	0.76	0.76	0.75	0.75
	H_o/H_e	1.03	1.00	1.03	1.01	1.00	1.00	1.00
	Overall F_{IS}	-0.023(0.88)	0.009(0.29)	-0.028(0.95)	-0.012(0.78)	-0.008(0.62)	-0.001(0.55)	0.002(0.48)
	N_e	7.5×10^3	7.1×10^3	7.9×10^3	7.9×10^3	7.9×10^3	7.5×10^3	7.5×10^3

[*1] Calculated according to the fomula: $a_e=1/\Sigma x_i^2$ (Crow and Kimura, 1965)

[*2] Probability value associated with F_{IS} is shown in parentheshis. F_{IS} values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ with initial K of sequential Bonferroni correction (Rice,1989), $K=11$.

[*3] Calculated according to the formula: $N_e=(H_e/(1\text{-}H_e))/4\mu$, where μ represents the microsatellite mutation rate (Crow and Kimura, 1970). The mutation rate is approximated as $\mu=10^{-4}$.

[*4] p is the exact p-value estimated by a test analogous to the Fisher's exact test in the Markov-chain method. Significant HWE departures for each locus determined by adding *=$p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

π values between populations was not pronounced (Table 8). All populations showed very high levels of haplotype diversity (h) ranging from 0.994 to 0.998, and the regional differences of the h values were also not significant.

Population differentiation and relationships

Table 9 shows the results of the pairwise heterogeneity test based on the microsatellite data. Four of the 11 loci, *Po33, Po52, Po56*, and *Po91*, showed significant differences of allele frequency distributions for at least one population pair (11 simultaneous tests, $p<0.005$). None of the remaining seven loci (*Po1, Po13, Po25A, Po26, Po35, Po42*, and *Po48*) showed significant genetic heterogeneities for all population pairs. Pairwise F_{ST} values varied depending on the locus with a maximum 0.077, which was observed at the *Po52* locus for a population pair of the HKD and NSK population. Significance of the F_{ST} values was observed at five of 11 loci for at least one population pair (11 simultaneous tests, $p<0.005$). Expression of these results in another manner revealed that each population showed significant genetic heterogeneities between one or more populations for at least one locus, and 11 of the 21 pairwise population comparisons yielded significant genetic heterogeneities. The pairwise Φ_{ST} values estimated based on the mtDNA sequence data were however significant at just three population pairs (HKD-NGT, HKD-IWT, and HKD-HYG population pairs) (Table 10).

Genetic distances (D_A and D_{ST}) between populations estimated based on microsatellite allele frequencies are summarized in Table 11, and Fig. 4 shows two types of unrooted NJ-trees reconstructed based on the D_A and D_{ST} distance measures. According to the tree derived from the D_A distance (D_A-NJ tree), it was possible to consider that the CHB, IWT, and HYG populations were clustered as one group and the remaining four populations, HKD, NGT, TTR, and NSK populations were clustered as another group, although it should be emphasized that the tree topology appears not to be highly robust given the short branch length between the groups and low bootstrap values obtained. The tree topology derived from the D_{ST} distance (D_{ST}-NJ tree) was in near agreement with the D_A-NJ tree topology except for the positions of the HKD population: this population was clustered with the TTR population in the D_A-NJ tree, whereas it was placed with the HYG population in the D_{ST}-NJ tree. As Takezaki and Nei (1996) described, this inconsistency might be due to the extent of sampling errors, which is an important factor for efficiency of a distance measure in reconstruction of phylogenetic tree. These authors also proposed that the D_{ST} distance is not so efficient in tree topology construction since it has a relatively large sampling error.

Several hierarchical AMOVA tests were conducted according to the population relationships estimated from the neighbor joining analysis. First, a putative group including all seven populations was defined, assuming that none of the seven populations within the group were structured. In this case, the fixation index was small but significantly differed from zero ($F_{ST}=0.0025$, $p=0.001$), suggesting that there

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    | → tRNA^Pro (71 bp)                                                    ←|
1   TCAGAAAAAGGAGATTTCAACTCCTACCCCTAACTCCCAAAGCTAGGATTCTAGCGTTAAACTATTTTCTG

    | → control region (≒372 bp)
72  GGAACATATGT T T TATGAAAATTAATATACATATATGTAAT TACACCATATAT T TATAGTAAACAT TAAGTCCGATGTACAAGACACAAAT GGATG TGAA
172 CAAAACATGGTGTCAAACAT TCATATACCAGC TATATAACTAAATATGTACAAAAACCAAACC TATAAGGTATACGATAAAGAAT TGAAGACTAATCGAA
272 ACTTTACACCGAACACAACCTTCATATGTCAAGTTATACCAAGACTCAAACCTCTGTCGATCCCAAA - TTCCCAT GCAGTAAGAGCCTACCATCAGTTGA
372 T TACT TAATGCCAACGGTTATTGAAGGTGAGGGACAAAAAT TGTGGGGGTTTCACACAGTGAAC TAT TCCTG
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Fig. 3. Sequence including the tRNA^{Pro} gene (71 bp) and the left domain of the control region of Japanese flounder mtDNA. Variable sites found in at least one individual are indicated by adding dots, and the single nucleotide deletion/insertion is represented by a dash (-).

Table 7. Frequency of each nucleotide at each variable site in the Japanese flounder mtDNA sequences including the $tRNA^{Pro}$ gene and the left domain of the control region

Populations	Nucleotides	← $tRNA^{Pro}$ | Control region → Sequence No.																																										
		25	33	56	74	77	79	84	85	88	95	97	98	122	124	125	134	137	139	140	143	152	153	156	158	159	161	164	165	166	171	172	173	176	177	178	179	181	183					
HKD	A	-	50	1	50	48	50	-	-	1	50	-	50	-	-	-	41	-	40	48	-	48	30	-	-	50	48	-	50	-	47	-	50	47	1	49	-	-	14					
(*N*=50)	C	-	-	-	-	-	-	2	-	-	-	1	-	4	-	1	-	-	10	-	50	2	-	50	49	-	-	-	-	-	-	50	-	-	48	-	-	-	-					
	G	-	-	49	-	2	-	-	-	49	-	-	-	-	-	-	9	-	-	2	-	-	20	-	-	-	2	50	-	-	3	-	-	1	-	1	-	50	36					
	T	50	-	-	-	-	-	48	50	-	-	49	-	46	50	49	-	50	-	-	-	-	-	-	1	-	-	-	-	50	-	-	-	-	1	-	50	-	-					
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-					
NGT	A	-	50	-	50	49	50	-	-	-	50	-	50	-	-	-	36	-	29	49	-	48	19	-	-	50	47	1	49	-	50	-	49	48	-	50	-	-	24					
(*N*=50)	C	-	-	-	-	-	-	8	-	-	-	-	-	10	1	-	-	-	21	-	50	-	-	50	50	-	-	-	1	-	-	50	-	-	49	-	-	-	-					
	G	-	-	50	-	1	-	-	-	50	-	-	-	-	-	-	14	-	-	1	-	2	31	-	-	-	3	49	-	-	-	-	-	-	-	-	50	26					
	T	50	-	-	-	-	-	42	50	-	-	50	-	40	49	50	-	50	-	-	-	-	-	-	-	-	-	-	-	50	-	-	1	-	1	-	50	-	-					
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-					
TTR	A	-	68	-	69	68	68	-	-	-	68	-	68	-	-	-	51	-	48	67	-	68	39	-	-	69	66	-	69	-	64	-	69	65	-	69	-	-	28					
(*N*=69)	C	1	-	-	-	-	-	12	-	-	-	1	-	12	-	-	-	3	21	-	69	-	-	68	69	-	-	-	1	-	69	-	-	69	-	-	-	-					
	G	-	1	69	-	1	1	-	-	69	-	-	1	-	-	-	18	-	-	2	-	-	30	-	-	-	3	69	-	-	5	-	-	-	-	-	-	69	41					
	T	68	-	-	-	-	-	57	69	-	1	68	-	57	69	69	-	66	-	-	-	1	-	1	-	-	-	-	-	68	-	-	-	-	-	-	69	-	-					
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-					
NSK	A	-	70	1	70	69	69	-	-	-	70	-	70	-	-	-	58	-	50	70	-	67	43	-	-	70	68	-	70	-	66	-	70	69	-	69	-	-	23					
(*N*=70)	C	-	-	-	-	-	-	12	-	-	-	-	-	13	-	1	-	1	19	-	68	-	-	70	69	-	1	-	-	1	-	69	-	-	69	-	-	-	-					
	G	-	-	69	-	1	1	-	-	70	-	-	-	-	-	-	12	-	-	-	-	3	27	-	-	-	1	70	-	-	4	-	-	-	-	1	-	70	47					
	T	70	-	-	-	-	-	58	69	-	-	70	-	57	70	69	-	69	1	-	2	-	-	-	1	-	-	-	-	69	-	1	-	-	1	-	70	-	-					
	indels	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-					
IWT	A	-	45	-	44	44	45	-	-	-	45	-	45	-	-	-	29	-	27	45	-	45	23	-	-	45	40	-	45	-	42	-	45	43	-	44	-	-	18					
(*N*=45)	C	-	-	-	-	-	-	13	-	-	-	1	-	11	-	1	1	-	18	-	45	-	-	45	45	-	1	-	-	-	-	45	-	-	45	-	1	-	-					
	G	-	-	45	1	1	-	-	-	45	-	-	-	-	-	-	15	-	-	-	-	-	22	-	-	-	4	45	-	-	3	-	-	-	-	1	-	45	27					
	T	45	-	-	-	-	-	32	45	-	-	44	-	34	45	44	-	45	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	44	-	-					
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-					
CHB	A	-	71	-	71	71	71	-	-	-	70	-	70	-	-	-	60	-	46	69	-	68	35	-	-	71	66	-	71	-	68	-	69	68	-	69	-	1	28					
(*N*=71)	C	-	-	-	-	-	-	10	-	-	-	-	-	11	-	2	-	2	25	-	70	-	-	70	69	-	2	-	-	-	-	71	-	-	71	-	1	-	-					
	G	-	-	71	-	-	-	-	-	71	-	-	1	-	-	-	11	-	-	2	-	1	36	-	-	-	3	71	-	-	3	-	-	-	-	2	-	70	43					
	T	71	-	-	-	-	-	61	71	-	1	71	-	60	71	69	-	69	-	-	1	2	-	1	2	-	-	-	-	71	-	-	2	-	-	-	70	-	-					
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-					
HYG	A	-	44	-	44	44	43	-	-	1	44	-	44	-	-	-	31	-	28	43	-	41	20	-	-	43	41	-	44	-	43	-	41	43	-	42	-	-	22					
(*N*=44)	C	-	-	-	-	-	-	10	-	-	-	-	-	12	-	-	-	-	15	-	44	-	-	44	42	-	-	-	-	-	-	44	-	-	44	-	-	-	-					
	G	-	-	44	-	-	1	-	-	43	-	-	-	-	-	-	13	-	-	1	-	3	24	-	-	1	3	44	-	-	1	-	-	-	-	2	-	44	22					
	T	44	-	-	-	-	-	34	43	-	-	44	-	32	44	44	-	44	1	-	-	-	-	-	2	-	-	-	-	44	-	-	3	-	-	-	44	-	-					
	indels	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-					

Populations	Nucleotides	Sequence No.																																										
		184	185	186	188	191	192	194	195	197	202	203	204	208	209	213	214	215	216	217	218	219	220	221	225	230	231	232	233	234	235	236	237	238	239	240	241	242	244					
HKD	A	-	-	50	33	-	-	50	-	-	1	-	-	-	50	49	49	43	-	-	45	-	36	-	39	-	49	50	50	-	-	-	48		48	48	-	1	50						
	C	-	45	-	-	4	-	-	-	-	2	48	2	-	-	-	-	-	-	-	-	13	-	1	-	50		-	-	46	35		-	-	-	-	-	-	-						
	G	-	-	-	17	-	-	-	-	-	47	-	-	-	-	1	1	7	-	-	5	-	14	-	-	-	-	-			-	-	1		2	2	50	49	-						
	T	50	5	-	-	46	50	-	50	50	-	2	48	50	-	-	-	-	-	50	-	37	-	49	11	-	1	-	-	4	15	50	1	50		-	-	-	-						
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
NGT	A	-	-	50	44	-	-	49	-	-	4	-	-	-	50	50	50	48	1	-	50		38	-	28	-	49	50	48	-	-	-	50		47	46	1	5	50						
	C	-	43	-	-	3	-	-	1	-	-	49	-	-	-	-	-	-	-	1	-	22	-	4	-	49	-	-	-	50	42		-	1	-	-	-	-						
	G	-	-	-	6	-	-	1	-	-	46	-	-	-	-	-	-	2	-	-	-	-	12	-	-		-	-	2	-	-	-	-	-	3	4	49	45	-						
	T	50	7	-	-	47	50	-	49	50	-	1	50	50	-	-	-	-	-	49	-	28	-	46	22	1	1		-	-	8	50	-	49	-	-	-	-	-						
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
TTR	A	-	-	69	58	-	-	69	-	-	9	-	-	-	69	69	68	62	-	-	67	-	41	-	48	-	69	69	67	-	1	-	69	-	66	64	3	3	69						
	C	2	54	-	-	6	-	-	-	1	-	66	-	-	-	-	-	-	-	4	-	25	-	8		69	-	-	-	63	52	1	-	1	-	-	-	-	-						
	G	-	-	-	11	-	-	-	-	-	60	-	-	-	-	-	1	7	-	-	2		28	-	-	-	-	-	2	-	-	-	-	-	2	4	66	66	-						
	T	67	15	-	-	63	69	-	69	68	-	3	69	69	-	-	-	-	-	65	-	44	-	61	21	-	-	-	-	6	16	68	-	68	-	-	-	-	-						
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
NSK	A	-	-	70	68	-	-	70	-	-	6	-	-	-	70	69	68	62	-	-	65	-	49	-	50	-	70	70	70	-	-	-	70	-	68	65	2	1	70						
	C	-	61			4	1	-	2	1	-	69	1	-	-	-	-	-	-	1	-	23	-	5	-	70	-	-	-	65	59		-	1	-	-	-	-	-						
	G	-	-	-	12	-	-	-	-	-	64	-	-	-	-	1	2	8	-	-	5	-	21	-	-	-	-	-	-	-	-	-	-	-	2	5	68	69	-						
	T	70	9		-	66	69	-	68	69	-	1	69	70	-	-	-	-	-	69	-	47	-	65	20	-	-	-	-	5	11	70		69		-	-	-	-						
	indels	-	-	-															70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
IWT	A	-	-	45	36	-	-	45	-	-	4	-	-	-	44	45	44	44	-	-	44	-	33	-	27	-	45	45	45	-	-	-	45	-	45	40	-	-	45						
	C		42	-	-	4	-	-	-	1	-	43	-	-						1	-	21	-	7	-	45		-	-	43	39	-	-	1	-	-	-	-	-						
	G	-	-	-	9	-	-	-	-	-	41	-	-	-	1	-	1	1	-	-	1	-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	5	45	45	-					
	T	45	3	-	-	41	45	-	45	44	-	2	45	45	-	-	-	-	-	44	-	24	-	48	18	-	-	-	-	2	6	45	-	44	-	-	-	-	-						
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
CHB	A	-	-	70	51	-	-	71	-	-	8	-	-	-	70	71	69	66	-	-	66	-	53	-	46		71	69	70		-	-	71		69	67	1	2	69						
	C	1	63	-	-	5	-	-	-	-	1	70	2	1	-	-	-	-		1	-	32	-	4	-	71	-	-	-	67	60	1	-	1	-	-	-	-	-						
	G	-	-	1	20	-	-		-	-	62	-	-	-	1	-	2	5	-	-	5	-	18	-	-	-	-	-	1	-	-	-	-	-	2	4	70	69	1						
	T	70	8	-	-	66	71	-	71	71	-	1	69	70	-	-	-	-	-	70	-	39	-	67	25	-		2	-	4	11	70	-	70	-	-	-	-	1						
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						
HYG	A	-	-	44	39	-		44	-	-	7	-	-	-	44	44	44	41	-	-	44	-	33	-	27	-	44	43	44	-	-		44	-	42	39	2	2	44						
	C	1	34		-	1			1	1	-	43	1	-	-	-	-	-	-	-	-	17	-	3	-	44	-	-	-	44	35	-	-	1	-	-	-	-	-						
	G	-	-		5	-			-	-	37	-	-	-	-	-	-	3	-	-	-	-	11	-	-	-		-	-	-	-	-	-	-	2	5	42	42	-						
	T	43	10		-	43	44		43	43	-	1	43	44	-	-	-	-	-	44	-	27	-	41	17	-		1	-	9	44	-	43		-	-	-	-	-						
	indels	-	-		-	-			-	-	-	-	-	-	-	-	-	-	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						

Table 7. (continued)

| Populations | Nucleotides | Sequence No. | | |

Table 8. Variability of the Japanese flounder mtDNA sequences including the $tRNA^{Pro}$ gene and the left domain of control region

	Populations						
	HKD	NGT	TTR	NSK	IWT	CHB	HYG
Sample size	50	50	69	70	45	71	44
Number of variable sites	76	80	87	95	45	103	76
Number of substitutions	81	81	91	100	74	110	80
Transitions	70	70	80	85	67	95	69
Transversions	11	11	11	15	7	15	11
Number of indels	2	3	3	4	2	3	3
Number of haplotypes	48	45	64	62	44	65	39
Haplotype diversity*1 (h)	0.998	0.996	0.998	0.996	0.999	0.998	0.994
Nucleotide diversity*2 (π)	0.027	0.031	0.031	0.028	0.030	0.030	0.032

*1 Nei and Tajima (1981)
*2 Nei (1987)

would be some genetic structures within the group. Next, hierarchical AMOVA tests were conducted forming putative groups estimated from each NJ tree topology (Table 12: test panel TD_A, for group definition on the basis of the D_A-NJ tree topology; test panel: TD_{ST}, for group definition on the basis of the D_{ST}-NJ tree topology). The putative groups defined based on the D_A-NJ tree topology were as follows: a group including the HKD, NGT, TTR, and NSK populations (group A1) and another group including the IWT, CHB, and HYG populations (group A2). As shown in Table 12, the fixation index among groups (F_{CT}) was not significant in this case (F_{CT}=0.0009, p=0.197), whereas it was significant among populations within groups (F_{SC} =0.0020, p=0.013). The groups defined based on the D_{ST}-NJ tree topology were as follows: a group including the NGT, TTR, and NSK populations (group ST1) and another group including the HKD, IWT, CHB, and HYG populations (group ST2). In this case, both F_{CT} and F_{SC} were significant (F_{CT}=0.0017, p=0.027; F_{SC}=0.0015, p=0.007). These results suggested that the populations are still structured within at least one group in each test panel since the F_{SC} values estimated in both test panels were significant. Therefore, AMOVA tests were performed for each group (group A1, A2, ST1, and ST2), assuming that none of the populations within each group were structured. In these cases, the F_{ST} values estimated for two groups (A1 and ST2) were significant (group A1: F_{ST} =0.0031, p=0.004; group A2: F_{ST} =0.0000, p =0.461; group ST1: F_{ST}=0.0009, p=0.207; group ST2: F_{ST}=0.0020, p=0.046). Of note, if the HKD population was removed from the group A1 and ST2, the population components of group A1 were identical to those of the group ST1, and the population components of group ST2 were identical to those of the group A2. Therefore, it was possibly considered that the HKD population could be subdivided from other populations as one group, and then the seven populations were putatively assigned into three groups as follows: a group comprised of the HKD population, the ST1 group, and the A1 group. The results of the AMOVA test based on this group definition (test panel: TA) showed that the fixation index among groups was significant (F_{CT} =0.0028, p=0.014), whereas it was not significant among populations within groups (F_{SC} =0.0005, p=0.272). According to the NJ tree topologies coupled with the results of the hierarchical AMOVA tests, the seven populations were possibly assigned into three groups: a group including two populations in the Japan Sea, the NGT and TTR, as well as one population in the East China Sea, the NSK population; a group including three populations in the Pacific Ocean, the IWT, CHB, and HYG populations, and a group comprised of the HKD

Table 9. Pairwise F_{ST} values[*1] between the 7 populations at each locus above the diagonal and results of pairwise comparisons of allele frequency distributions[*2] below the diagonal

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po25A	HKD		0.002	0.017	0.001	0.000	0.005	-0.003
			(0.29)	(0.01)	(0.33)	(0.38)	(0.15)	(0.65)
	NGT	0.17		0.006	-0.007	-0.006	0.000	0.001
				(0.14)	(1.00)	(0.82)	(0.40)	(0.36)
	TTR	0.09	0.18		0.014	-0.001	0.000	0.027*
					(0.02)	(0.45)	(0.35)	(0.0017)
	NSK	0.20	0.99	0.053		-0.001	0.004	-0.003
						(0.47)	(0.14)	(0.64)
	IWT	0.38	0.89	0.34	0.61		-0.003	0.007
							(0.62)	(0.15)
	CHB	0.10	0.71	0.57	0.41	0.84		0.007
								(0.11)
	HYG	0.44	0.54	0.03	0.73	0.56	0.67	

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po56	HKD		0.008	0.001	0.012*	-0.005	0.008	-0.001
			(0.03)	(0.30)	(0.0018)	(0.96)	(0.01)	(0.57)
	NGT	0.01		-0.001	0.009	0.005	0.005	0.008
				(0.52)	(0.01)	(0.07)	(0.05)	(0.03)
	TTR	0.39	0.46		0.000	-0.004	-0.001	-0.002
					(0.40)	(0.90)	(0.61)	(0.67)
	NSK	0.0015*	0.01	0.19		0.004	-0.001	0.002
						(0.13)	(0.54)	(0.25)
	IWT	0.88	0.10	0.89	0.06		0.000	-0.006
							(0.39)	(0.99)
	CHB	0.0029*	0.02	0.35	0.15	0.47		-0.002
								(0.73)
	HYG	0.48	0.02	0.87	0.31	1.00	0.73	

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po33	HKD		-0.007	0.004	0.004	0.000	-0.001	0.024
			(0.83)	(0.19)	(0.19)	(0.34)	(0.45)	(0.03)
	NGT	0.34		0.001	0.009	0.008	0.004	0.038
				(0.32)	(0.10)	(0.14)	(0.20)	(0.01)
	TTR	0.01	0.26		0.006	0.005	0.004	0.041*
					(0.12)	(0.16)	(0.16)	(0.0021)
	NSK	0.61	0.37	0.07		-0.006	-0.001	0.008
						(0.84)	(0.45)	(0.12)
	IWT	0.21	0.08	0.06	0.93		-0.007	0.004
							(0.93)	(0.24)
	CHB	0.24	0.06	0.0022*	0.50	0.87		0.012
								(0.08)
	HYG	0.01	0.0011*	0.0000*	0.09	0.11	0.02	

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po91	HKD		0.005	0.000	0.000	0.011	0.007	0.004
			(0.09)	(0.42)	(0.46)	(0.01)	(0.02)	(0.13)
	NGT	0.12		0.001	0.002	0.016*	0.006	0.002
				(0.26)	(0.18)	(0.0013)	(0.03)	(0.30)
	TTR	0.32	0.34		-0.004	0.002	0.003	-0.001
					(0.97)	(0.21)	(0.13)	(0.55)
	NSK	0.47	0.26	0.87		0.001	-0.001	-0.003
						(0.31)	(0.55)	(0.85)
	IWT	0.0004*	0.0037*	0.37	0.22		0.001	0.002
							(0.38)	(0.28)
	CHB	0.02	0.20	0.01	0.15	0.25		-0.003
								(0.90)
	HYG	0.0044*	0.60	0.32	0.33	0.50	0.74	

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po52	HKD		0.010	0.054*	0.077*	0.004	0.004	0.006
			(0.14)	(0.0018)	(0.0000)	(0.20)	(0.23)	(0.24)
	NGT	0.06		0.011	0.023	-0.009	0.002	0.000
				(0.10)	(0.04)	(0.91)	(0.29)	(0.35)
	TTR	0.0004*	0.37		-0.005	0.019	0.048*	0.014
					(0.71)	(0.05)	(0.0015)	(0.09)
	NSK	0.0000*	0.10	0.85		0.033	0.067*	0.030
						(0.02)	(0.0000)	(0.02)
	IWT	0.10	0.89	0.12	0.04		-0.004	0.003
							(0.57)	(0.28)
	CHB	0.14	0.53	0.01	0.0012*	0.56		0.017
								(0.06)
	HYG	0.35	0.31	0.07	0.0040*	0.26	0.09	

Pop.	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD		NS	+	+	+	+	+
NGT			NS	NS	+	NS	+
TTR				NS	NS	+	+
NSK					NS	+	+
IWT						NS	NS
CHB							NS
HYG							

+: population pair showing significant genetic heterogeneities for at least one locus

NS: population pair not showing any significant genetic heterogeneities for all loci

[*1] F_{ST} values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ with initial K of sequential Bonferroni correction (Rice, 1989), $K=11$. The probability for each comparison is also shown in parenthesis.

[*2] Probability values of homogeneity of allelic frequency distributions (p) estimated by a test analogous to the Fisher's exact test in the Markov-chain method are shown. Significance for each pairwise comparison was determined by adding *=$p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

Six loci, *Po1, Po13, Po26, Po35, Po42*, and *Po48*, did not show any significant genetic heterogeneities of both allele frequency distributions and F_{ST} values for all population comparisons ($p>0.005$)

Table 10. Number of mtDNA haplotypes commonly shared between the 7 populations below the diagonal, and pairwise Φ_{ST} values[*1] estimated based on the mtDNA sequences above the diagonal

	Populations						
Populations	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD	—	0.043**	0.0121	0.0059	0.0399*	0.0099	0.0286*
		(0.007)	(0.076)	(0.168)	(0.010)	(0.099)	(0.023)
NGT	5	—	0.0080	0.0142	-0.0027	0.0048	-0.0097
			(0.135)	(0.071)	(0.450)	(0.188)	(0.878)
TTR	5	4	—	-0.0027	0.0032	-0.0024	-0.00232
				(0.556)	(0.247)	(0.548)	(0.471)
NSK	7	7	7	—	0.0064	-0.0040	0.0044
					(0.169)	(0.697)	(0.213)
IWT	5	5	10	9	—	0.0009	0.0001
						(0.320)	(0.345)
CHB	3	4	9	9	3	—	0.0005
							(0.343)
HYG	3	2	5	5	5	3	—

[*1] Calculated according to Weir and Cockerham (1984). Probability values associated with the Φ_{ST} values are also shown in parenthesis. Significance for each pairwise comparison was determined by adding *=$p<0.05$, and **=$p<0.01$.

Table 11. Modified Cavalli-Sforza chord distances (D_A) between the 7 populations above the diagonal and Nei's standard genetic distance (D_{ST}) below the diagonal

	Populations						
Populations	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD	—	0.083	0.072	0.076	0.088	0.083	0.090
NGT	0.011	—	0.061	0.061	0.073	0.066	0.078
TTR	0.017	0.004	—	0.051	0.063	0.061	0.075
NSK	0.025	0.007	0.000	—	0.064	0.061	0.072
IWT	0.008	0.006	0.000	0.000	—	0.056	0.074
CHB	0.014	0.010	0.007	0.009	0.000	—	0.068
HYG	0.014	0.011	0.016	0.003	0.001	0.008	—

population.

The mtDNA sequence data, however, did not support the results derived from the microsatellite data: for the global analysis (including all populations with no subdivision) associated with the Φ_{ST} distance, there was not sufficient evidence to consider that the seven populations were structured ($\Phi_{ST}=0.0062$, $p=0.063$), and also, a hierarchical AMOVA test carried out according to the microsatellite-based group definition did not yield any significant heterogeneities among the groups ($\Phi_{CT}=0.0037$, $p=0.258$).

Discussion

Genetic variability

Genetic variability of the 11 microsatellites in terms of the allelic diversity (A: 15.3-18.2) and the gene diversity (mean H_e value: 0.74-0.76) were roughly similar to those reported in other marine fish microsatellites, and were obviously higher than those observed in allozyme loci (see Chapter 2). Given that the Mendelian inheritability for each of the 11 loci was verified (Chapter 2), and that the H_o values were well in accordance with the H_e values in each

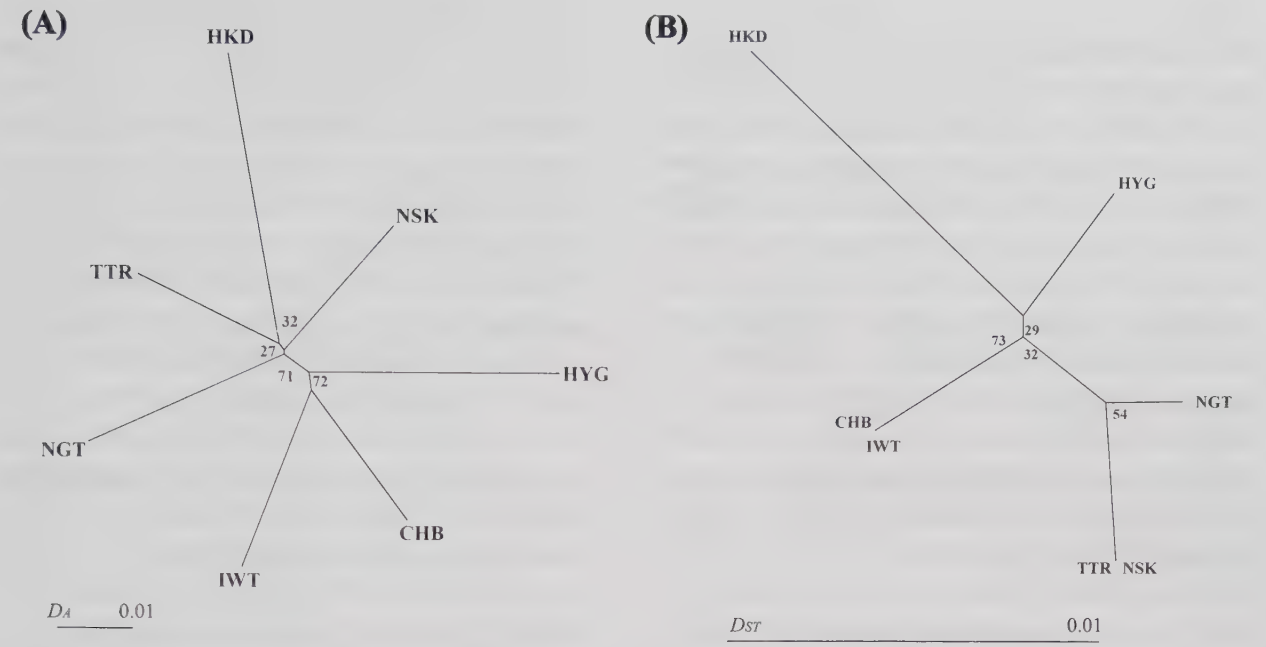

Fig. 4. (A) Unrooted neighbor-joiningtree based on modified Cavalli-Sforza chord distance (D_A; Nei *et al.*, 1983); (B) Unrooted neighbor-joining tree based on Nei's standerd genetic distance (D_{ST}; Nei, 1972). The numbers refer to percentage bootstrap values generated from 1,000 replications of re-sampled loci.

Table 12. Results of hierarchical AMOVAs for groups having several combinations of populations

Test panels*1	Number of groups	Group definitions	Among groups (F_{CT})	Among populations within group (F_{SC})	Within populations (F_{ST})
TD_A	2	(HKD-NGT-TTR-NSK)	0.0009	0.0020*	0.0028*
		vs (IWT-CHB-HYG)	(0.197)	(0.013)	(0.001)
TD_{ST}	2	(NGT-TTR-NSK)	0.0017*	0.0015*	0.0032*
		vs (HKD-IWT-CHB-HYG)	(0.027)	(0.007)	(0.001)
TA	3	HKD *vs* (NGT-TTR-NSK)	0.0028*	0.0005	0.0033*
		vs (IWT-CHB-HYG)	(0.014)	(0.272)	(0.001)

*1 See text for details

Probability values associated with *F*-statistics are shown in parenthesis. *F*-statistics significantly greater than zero, based on random allelic permutation testing, are noted by adding $*=p<0.05$.

population at each locus, a possible presence of null alleles seems unlikely to be a major concern for the 11 loci. It is also unlikely that the significant HWE departure observed at the *Po42* locus in the IWT population was caused by several possible factors such as inbreeding, assortative mating or Wahlund effects since all loci but the *Po42* locus did not show any significant HWE departures in this population. Alternatively, it would be plausible to consider that the HWE departure was caused by random variations.

An average of the effective population size (N_e) among the seven populations was estimated to be of 7,600. This value was comparably lower than that reported in other marine fish species such as the vermilion snapper ($N_e=24{,}500$; Bagley *et al.*, 1999) and European sea bass ($N_e=11{,}000$-$15{,}000$; Garcia de León *et al.*, 1997), although the estimate of N_e was calculated based on several assumptions (*e. g.*, microsatellite mutation rates and mutation models), and thus the N_e value may entail a wide range of errors.

The mtDNA control region of Japanese

flounder has been shown to be highly variable (Fujii and Nishida, 1997), and as well this study also bore out the large amounts of nucleotide variations in the control region, in which 147 variable sites among 399 individuals were detected. A high level of nucleotide variations maintained in the control region was also found in other marine fish species such as the swordfish (Alvarado Bremer *et al.*, 1995) and red sea bream (Tabata and Taniguchi, 2000). A selective constraint operates on mutations in coding regions, possibly having an effect on the mutation rates (Carvalho and Hauser, 1994). The mtDNA control region is however generally known to be a non-transcribed region, and hence it is likely that the neutrality of the mtDNA control region may allow itself to be highly variable. The high levels of mtDNA and microsatellite variabilities detected in the Japanese flounder indicate that a severe stock depression resulting in population bottlenecks has not occurred for the long term evolutionary history of wild Japanese flounder populations.

Population differentiation and relationships

The first point that should be discussed about the Japanese flounder genetic structure is that the results derived from the microsatellite and mtDNA data were inconsistent with each other. The overall F_{ST} value estimated from the microsatellite data showed a significant genetic structure among the seven populations (overall $F_{ST}=0.0025$, $p=0.001$), however, the mtDNA data did not reject the null hypothesis of overall genetic homogeneity among the populations (overall $\Phi_{ST}=0.0062$, $p=0.063$). A previous allozymic analysis (Fujio *et al.*, 1989) and PCR-RFLP of mtDNA (Asahida *et al.*, 1998) also failed to detect any significant genetic heterogeneities between several regional populations, although it seems likely that the population homogeneity revealed by the previous studies are largely accounted for by the limited number of available polymorphic loci of allozymes (2 of the 23 loci examined were available as polymorphic markers), and the small a number of individuals examined in the PCR-RFLP analysis (up to 10 individuals in each population). It is widely recognized that different parts of the genomes have passed different evolutionary processes (Avise, 1994; Huelsenbeck *et al.*, 1996), possibly leading to the existence of different mutation rates between the nuclear and mitochondrial genomes. Nielsen *et al.* (1997) assessed the population structure of Pacific trout in California and Mexico using the nucleotide sequences of mtDNA control region coupled with microsatellites, and they also found an incongruity of genetic structure between the two molecular markers. These authors inferred that the incongruity would be caused by the different mutation rates between the microsatellites and mtDNA. Although the results presented here are not enough to determine the causes of the conflicting results derived from the two molecular markers, microsatellites distribute ubiquitously throughout the nuclear genome, and it is thus conceivable that the microsatellite data would better represent the entire genomic information than the mtDNA data, which are derived from a small portion (approximately 440 bp) of the control region. The present author therefore considers that the microsatellites serve as a more appropriate marker to assess the genetic structure, rather than the sequences of mtDNA control region. Here, it is useful to look more closely at the significant genetic structure found in the microsatellite data.

None of the 11 microsatellites showed significant genetic heterogeneities between the three populations located in areas affected by the Tsushima Warm Current (the NGT, TTR, and NSK populations), and between the three populations in the Pacific Ocean (the IWT, CHB, and HYG populations) in terms of both allele frequency distributions and pairwise F_{ST} values. Japanese flounder larvae are possibly transported approximately 600km or more by the Tsushima Warm Current (Kinoshita *et al.*, 2000), and this prevailing oceanic current may homogenize the allele frequencies among populations from the Japanese coast side of the East

China Sea through the Japanese coast side of the Japan Sea. Little is known about the mode and extent of larvae dispersal in the Pacific Ocean, it is also probable that the Oyashio Cold Current and the Kuroshio Warm Current, which mixes with the Oyashio Cold Current, may effectively mix the genetic components among populations throughout the Japanese coast side of the Pacific Ocean. Therefore, these genetic similarities among populations are due most likely to the frequent occurrence of inter-populational gene flow by means of eggs and larvae transport as provided by the prevailing oceanic currents. While, Kinoshita *et al.* (2000) proposed population subdivisions in the Japan Sea from morphological points of view; these authors found geographic variation in dorsal and anal ray counts of juvenile Japanese flounder with significant differences between northern and southern groups in the Japan Sea. According to the geographical areas surveyed together with group definitions proposed by these authors, the NGT and TTR populations are assigned to the northern and southern groups, respectively. The present study did not support the findings inferred from the morphological study: neither the microsatellite data nor the mtDNA sequence data yielded significant genetic heterogeneities among populations within the areas for which they surveyed.

The estimate of F_{ST} among the seven populations was indeed significantly different from zero but it was very small ($F_{ST}=0.0025$, $p=0.001$), indicating that Japanese flounder populations around Japan are not well-structured. Even if the NJ tree topologies coupled with the results of AMOVA tests could illustrate the outline of population relationships, that is, it might be accounted for by oceanographical separation, the small F_{ST} value estimated among the populations and the low robustness of the NJ tree topologies indicate a very weak genetic integrity among populations. The mtDNA sequencing analysis employed in this study failed to detect this subtle genetic population differentiation. A geographical barrier such as the Japanese Archipelago separating the two oceanographic areas (the Japan Sea and the Pacific Ocean) seems not to be effective to maintain genetic population integrity between the two areas. This is indicated by the facts that several population pairwise comparisons such as IWT-TTR, IWT-NSK, and CHB-NGT population pairs did not yield any genetic heterogeneities for all microsatellites, despite that the two populations in each population pair were separated from one another by the Japanese Archipelago. The mtDNA sequence data also indicate the weak genetic integrity between the areas since population pairs between the two populations in the Pacific Ocean (the IWT and CHB populations) and the two populations in the Japan Sea (the TTR and NSK populations) shared a comparably large number of common mtDNA haplotypes (9-10 haplotypes). Bagley *et al.* (1999) reported the absence of genetic heterogeneities among vermilion snapper populations off the southeastern USA using several microsatellite markers, and these authors described that level of gene exchange in terms of N_em (where N_e is the effective population size, and m is the inter-populational migration rate per generation under the infinite island model) estimated among the populations between the South Atlantic Bight and the Gulf of Mexico was sufficient to prevent genetic population differentiation between the two oceanographic areas ($N_em>100$). According to the formula $N_em\fallingdotseq(1/F_{ST}-1)/4$ (Slatkin, 1985) together with the $F_{ST}=0.0025$ estimated for Japanese flounder, the number of migrants can be roughly estimated to be of $N_em\fallingdotseq100$, which is a similar order of magnitude as estimated for the vermilion snapper. Given the average of N_e values estimated for Japanese flounder ($N_e=7{,}600$), the migration rate (m) among populations is estimated to be of 0.013 (1.3%) per generation. This value is sufficiently high to counteract a significant genetic drift: according to a simulation analysis studied by Lacy (1987), the effect of genetic drift in an isolated population, in which 120 individuals are included, will be diminished if one immigrant arrives every generation in

the population (m=0.008).

The genetic structure of wild Japanese flounder could be summarized as follows: overall, gene flows would occur frequently from one area to adjacent areas resulting in the subtle inter-populational genetic differentiation, but not occur with an equal probability between areas as this study provided evidence of the significant genetic heterogeneity among the three groups. It is possible to consider that the genetic heterogeneity among the groups has been kept in the evolutionary history of Japanese flounder populations; however, further analyses for other populations with extended period of time should be conducted to depict the genetic structure of Japanese flounder in finer scale.

Summary

As discussed above, genetic integrity among wild Japanese flounder populations around Japan is not pronounced; nevertheless the present author suggests that the Japanese flounder should not be treated as an overall panmictic population. This is an important finding in this study since almost all of the previous genetic studies have failed to detect any significant genetic population heterogeneities around Japan (but see Fujii and Nishida, 1997). Even if the extent of the genetic population differentiation is subtle, wild Japanese flounder populations should not be managed as a single stock unit; Brown *et al.* (1987) studied the exchange rate between yellowtail flounder stocks, and these authors reported that two stocks with an exchange rate of 10% (m=0.10), which is approximately ten-fold higher than that estimated for among Japanese flounder populations (m=0.013), still reacted independently to the exploitation. The stocking practices of Japanese flounder should be conducted along with the concept of stock-conservation taking into consideration the genetic stock units. The present author recommends that the genetic management of wild Japanese flounder populations, if possible, be executed at every local area. The extent of genetic stock units that should be managed, however, can be extended by within each geographical area in which each of the three groups defined in this study is comprised.

Chapter 4. Assessment of genetic diversity within and between hatchery strains of Japanese flounder

The preceding chapter revealed that natural Japanese flounder populations possess a high amount of genetic variations. On the other hand, loss of genetic variation in hatchery strains is typical, and it is very likely that the reduced genetic variabilities are caused by the small number of founders and/or inbreeding events that occurred when the strains were founded, as clearly shown by recent direct DNA examinations (*e. g.*, Atlantic salmon, Norris *et al.*, 1999; ayu fish, Iguchi *et al.*, 1999). Regarding the Japanese flounder hatchery strains, genetic assessment has been employed using several classes of molecular markers. An allozymic analysis (Liu *et al.*, 1997) found significant genetic differentiation between several hatchery strains, and also between hatchery strains and wild populations; however, allozyme loci did not clearly show the reductions of genetic variability in the hatchery strains, this seemed to be due to the limited number of available polymorphic loci and the low levels of allelic diversity. More powerful techniques such as mitochondrial DNA (mtDNA)-RFLP (Sugaya *et al.*, 1999) and microsatellite analyses (Yoshida *et al.*, 2000) detected significant reductions of genetic variability in several hatchery strains. The molecular markers employed in these studies were found to be appropriate to detect reduced genetic variability in hatchery strains; nevertheless the present author considers that a simultaneous use of mtDNA and nuclear DNA-based markers such as microsatellite markers can clearly demonstrate a loss of genetic variations in hatchery strains.

This chapter presents an application of two

classes of molecular markers, that is, microsatellites and nucleotide sequences of mtDNA control region, to the hatchery strains of Japanese flounder derived from three hatchery stations. Of the hatchery strains examined, two strains were first-generation offspring of wild caught fish, while the other strain was founded using both wild and broodstock, which were hatchery-reared potentially over several generations. It should be noted that a loss of genetic variability might possibly occur even in the first hatchery generation (Verspoor, 1988). This chapter aims to deduce the genetic diversity within and between the hatchery strains to be stocked into the natural environment, documenting the potential uses of the two molecular markers for further monitoring of genetic conditions in hatchery strains.

Materials and methods

Fish samples

Hatchery fish were provided from a hatchery station in Hokkaido Prefecture (abbr. HY; 100 individuals), in Tottori Prefecture (abbr. HF; 100 individuals), and in Miyagi Prefecture (abbr. HC; 100 individuals). The HY strain was founded using approximately 110 wild caught flounder including 50 females and 60 males which were sampled from off Hokkaido Prefecture, and all fish in this strain were F_1 offspring of the wild captives. The HF strain was founded using approximately 300 individuals: 100 individuals including 50 females and 50 males were mated in each of three aquarium tanks, and the offspring sampled from each tank were communally reared in a single tank. The candidate broodstock were both wild caught flounder sampled from off Tottori Prefecture and fish maintained potentially over several generations in this hatchery. Unfortunately, there are no available records regarding how many of the sibs were used for the founding of this strain. The HC strain originated from approximately 60 wild flounder including 30 females and 30 males caught in off Miyagi Prefecture, and this strain comprised of F_1 offspring of the wild captives. Genetic information of the candidate broodstock for all hatchery strains was not available.

The genetic variability in each of the three hatchery strains was measured against that of the geographically proximal wild population examined in Chapter 3, that is, the HY strain was compared with the HKD population, the HF strain with the TTR population, and the HC strain with the CHB population.

Microsatellite and mitochondrial DNA sequencing analysis

Eleven microsatellites described in Chapter 2, *Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48, Po52, Po56*, and *Po91*, and a section of the mtDNA control region (see the preceding chapter), were screened for all fish samples. Microsatellite genotyping and mtDNA sequencing procedures are described in the preceding chapter.

Microsatellite allele frequencies and gene diversity (unbiased expected heterozygosity: H_e) of each strain at each locus were estimated using the ARLEQUIN version 1.1 software package (Schneider *et al.* 1997). Differences of mean H_e values between the hatchery strains and wild populations were tested using the Kruskal-Wallis rank sum analysis (Sokal and Rohlf 1997). The ARLEQUIN program was used for an assessment of the Hardy-Weinberg equilibrium (HWE) at each locus using a test analogous to the Fisher's exact test using the Markov-chain method (the Markov-chain parameters used were: steps, 100,000; dememorization, 10,000). An overall inbreeding coefficient (F_{IS}; Weir and Cockerham 1984) was also estimated to measure the HWE departures evaluating the probabilities through random permutation procedures (minimum 10,000 permutations). The value of significance associated with the HWE analysis was adjusted following sequential Bonferroni procedures (Rice 1989).

As regards the mtDNA sequence data, the number of variable sites, haplotype frequency distributions, and haplotype diversity (h) were calculated using the ARLEQUIN program. The

h values were compared between the hatchery strains and wild populations by the Kruskal-Wallis analysis.

Overall F-statistics (Weir and Cockerham, 1984) were estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}) using the ARLEQUIN program to assess the strain/population differentiation. Probability associated with the F_{ST} (Φ_{ST}) values was evaluated through random permutation procedures (minimum 10,000 permutations).

Results

Microsatellite variability

Allele frequency distributions for the three hatchery strains at each locus are listed in Appendix 2, and Table 13 summarizes the variabilities with the comparable wild population data. In the three hatchery strains, the allelic diversity (number of alleles per locus: A) ranged from 5.9 to 10.7, and the mean H_o and H_e values ranged from 0.57 to 0.72, and from 0.59 to 0.71, respectively. The A values estimated for the three hatchery strains were substantially reduced compared with those estimated for the three wild populations (15.3-18.2), despite the fact that the sample size of each hatchery strain (100 individuals) was larger than that of each wild population (50-72 individuals). Comparison of the mean H_e values revealed that the values estimated for the hatchery strains (H_e=0.59-0.71) were significantly lower than those estimated for the wild populations (H_e=0.75-0.76) (Kruskal-Wallis test, 1df, H=29.6, $p<0.01$). The HF strain showed a marked reduction of H_e value compared to all other strains/populations. There appeared not to be large discrepancies between the H_o and H_e values for almost all of the 11 loci, the *Po42* locus, however, showed a significant depression of heterozygous individuals compared with the expected ones in the HF strain (H_o=0.56 and H_e=0.72). Significant HWE departures were observed in all hatchery strains depending on the locus (11 simultaneous tests, $p<0.005$). This is in contrast to the results of the wild populations analyzed: the 11 loci did not show any significant HWE departures in all the three wild populations (Table 13). Negative values of overall inbreeding coefficients (F_{IS}) were estimated excepting one instance: a comparably higher F_{IS} value was estimated for the HF strain (F_{IS}=0.018), although the value was not significantly different from zero (p=0.20).

Mitochondrial DNA sequence variability

Within approximately 443 bp sequences containing the $tRNA^{Pro}$ gene (71 bp) and the left domain of the control region of mtDNA, a total of 48 variable sites were detected among the 300 individuals in the three hatchery strains. Haplotypic variabilities of the three hatchery strains are summarized in Table 14 with the wild population data. A total of 25 haplotypes were identified across the three hatchery strains, whereas 160 haplotypes were identified among 190 individuals in the three wild populations. The small number of haplotypes identified in hatchery strains (4-14 haplotypes; see also Fig. 5) was in contrast to the large a number of haplotypes observed in the wild populations (48-66 haplotypes). The three hatchery strains did not share any common haplotypes with each other, while several haplotypes were commonly shared between the hatchery strains and the wild populations (Table 15). The hatchery strains had a significantly lower haplotype diversity (h=0.692-0.798) than that estimated for the wild populations (h=0.998 for all the wild populations) (Kruskal-Wallis test, 1df, H=33.5, $p<0.01$). The HF strain, in which the microsatellite variability was substantially reduced, also showed an extremely low haplotype variability.

Strain/population differentiation

Overall F-statistics were estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}), and Table 16 shows the values estimated for the various sample combinations. A high level of genetic differentiation with statistically significant F_{ST} (Φ_{ST}) was estimated among all samples (no subdivisions), and among the

Table 13. Microsatellite variabilities of the 11 loci in the 3 hatchery strains and 3 wild populations of Japanese flounder

Locus		Hatchery strains			Wild populations		
		HY	HF	HC	HKD	TTR	CHB
Po1	Sample size	100	100	100	50	69	72
	$A(a_e$ [*1])	12(3.0)	5(1.5)	12(4.8)	24(7.7)	26(5.6)	28(5.3)
	H_o	0.67	0.30	0.84	0.86	0.87	0.81
	H_e	0.67	0.33	0.79	0.87	0.82	0.81
	p [*2]	0.000*	0.021	0.385	0.35	0.63	0.37
Po13	Sample size	100	100	100	50	69	72
	$A(a_e)$	15(6.7)	8(2.4)	15(9.1)	25(16.7)	27(12.5)	26(16.7)
	H_o	0.85	0.56	0.96	0.90	0.97	0.97
	H_e	0.85	0.58	0.89	0.94	0.92	0.94
	p	0.000*	0.001*	0.004*	0.07	0.99	0.09
Po25A	Sample size	100	100	100	50	69	71
	$A(a_e)$	13(6.7)	6(4.5)	7(4.3)	11(5.9)	12(4.2)	15(5.6)
	H_o	0.91	0.81	0.79	0.74	0.77	0.79
	H_e	0.85	0.78	0.77	0.83	0.76	0.82
	p	0.000*	0.01	0.31	0.05	0.31	0.36
Po26	Sample size	100	100	100	50	68	72
	$A(a_e)$	3(2.9)	3(2.8)	3(2.1)	6(2.6)	5(2.9)	5(3.0)
	H_o	0.71	0.72	0.58	0.68	0.74	0.67
	H_e	0.65	0.64	0.52	0.62	0.65	0.67
	p	0.04	0.04	0.06	0.90	0.69	0.74
Po33	Sample size	100	100	100	50	69	72
	$A(a_e)$	7(3.6)	7(3.3)	5(2.3)	7(2.5)	10(3.1)	9(2.7)
	H_o	0.75	0.67	0.60	0.68	0.72	0.58
	H_e	0.72	0.70	0.57	0.60	0.68	0.63
	p	0.14	0.34	0.50	0.47	0.61	0.02
Po35	Sample size	100	100	100	50	69	71
	$A(a_e)$	12(5.9)	8(4.0)	11(2.9)	17(5.0)	19(4.5)	18(5.3)
	H_o	0.87	0.77	0.61	0.84	0.81	0.81
	H_e	0.83	0.75	0.66	0.80	0.78	0.81
	p	0.000*	0.46	0.01	0.45	1.00	0.14
Po42	Sample size	100	100	100	50	69	71
	$A(a_e)$	15(6.3)	7(3.6)	15(7.7)	23(10.0)	23(11.1)	28(14.3)
	H_o	0.83	0.56	0.85	0.96	0.88	0.93
	H_e	0.84	0.72	0.87	0.90	0.91	0.93
	p	0.000*	0.000*	0.000*	0.87	0.64	0.33
Po48	Sample size	100	100	100	50	64	72
	$A(a_e)$	5(2.2)	2(1.7)	6(2.4)	6(2.0)	7(1.9)	9(1.9)
	H_o	0.53	0.36	0.65	0.54	0.46	0.47
	H_e	0.55	0.39	0.59	0.50	0.46	0.48
	p	0.000*	0.60	0.67	0.49	0.74	0.34
Po52	Sample size	100	100	100	50	69	72
	$A(a_e)$	3(1.1)	2(1.1)	3(1.2)	4(1.5)	4(2.0)	4(1.5)
	H_o	0.06	0.05	0.20	0.34	0.46	0.33
	H_e	0.06	0.05	0.19	0.34	0.50	0.32
	p	1.00	1.00	0.69	0.88	0.60	0.66
Po56	Sample size	100	100	100	50	69	71
	$A(a_e)$	16(8.3)	7(3.7)	18(12.5)	22(16.7)	26(16.7)	23(14.3)
	H_o	0.86	0.72	0.94	0.96	0.94	0.93
	H_e	0.88	0.73	0.92	0.94	0.94	0.93
	p	0.000*	0.001*	0.05	0.49	0.77	0.95
Po91	Sample size	100	100	100	50	69	72
	$A(a_e)$	17(7.7)	10(4.3)	15(8.3)	23(14.3)	34(16.7)	35(20)
	H_o	0.91	0.80	0.92	0.94	0.96	0.96
	H_e	0.87	0.77	0.88	0.93	0.94	0.95
	p	0.000*	0.004*	0.003*	0.98	1.00	1.00
Mean	$A(a_e)$	10.7(3.4)	5.9(2.4)	10(3.3)	15.3(4.0)	17.5(4.2)	18.2(4.0)
	H_o	0.72	0.57	0.72	0.77	0.78	0.75
	H_e	0.71	0.59	0.70	0.75	0.76	0.75
	Overall $F_{IS}(p)$ [*3]	− 0.021(0.86)	0.018(0.20)	−0.037(0.98)	−0.023(0.88)	− 0.028(0.95)	−0.001(0.55)

[*1] a_e: effective number of alleles was calculated according to the formula: $a_e = 1/\Sigma x_i^2$ (Crow and Kimura, 1965)

[*2] p is the exact p-value estimated by a test analogous to Fisher's exact test in the Markov-chain method. Significant HWE departure for each locus was determined by adding $*=p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

[*3] Probability value associated with the F_{IS} is shown in parenthesis. The F_{IS} values significantly greater than zero, based on random allelic permutation testing, are noted by adding $*=p<0.005$ with initial K of sequential Bonferroni correction (Rice, 1989), $K=11$.

hatchery strains ($p<0.001$). These values were higher than those estimated for among the wild populations. Each hatchery strain was compared with the geographically proximal wild population (*i. e.*, the HY strain with the HKD population; the HF strain with the TTR population; the HC strain with the CHB population). These sample combinations were expected to minimize differences between the hatchery strains and wild populations caused by any regional differences in frequencies of microsatellite alleles and mtDNA haplotypes. The F_{ST} and Φ_{ST} values estimated for all sample combinations were significantly different from zero in these cases ($p<0.001$). The *F*-statistics were also estimated for between the hatchery group into which the three hatchery strains were pooled and the wild group into which the three wild populations were pooled. The estimate of F_{ST} and Φ_{ST} was also significant in this case ($p<0.001$). As was reported in brown trout populations (reviewed in Ferguson *et al.*, 1995), it appears that the Φ_{ST} values estimated from the mtDNA data were approximately double the F_{ST} values estimated from nuclear DNA (microsatellites) data with the exception of one instance (for a comparison among wild populations).

Discussion

Genetic variability

Microsatellite variability of the hatchery strains was characterized as substantial reductions of allelic diversity in terms of the number of alleles per locus. This would be caused by the loss of many low frequency alleles due most likely to the small number of effective parents when each strain was founded, suggesting that each strain was bottlenecked. Allelic diversity has been shown to be variable depending on the sample size, although the sample size of all the three hatchery strains screened in this study was larger than those for the wild populations. While, the mtDNA genome is theoretically transmitted thorough a single maternal line with no recombination, and mtDNA variabilities are thus expected to be highly susceptible to stochastic events such as population bottlenecks (Carvalho and Hauser, 1994). In practical terms, there were marked reductions as regards both the number of mtDNA haplotypes and haplotype diversity in the hatchery strains, consistent with population bottlenecks. With the facts that the large number of haplotypes were observed in the wild populations (160 haplotypes among 190 individuals), and the HY and HC strains were the first generation of wild caught flounder, it is reasonable to assume that the number of haplotypes detected in the HY strain (14 haplotypes) and the HC strain (7 haplotypes) represents the actual number of female parents by which each strain was founded. Given that the HY strain was founded using approximately 50 females and the HC strain using 30

Table 14. MtDNA sequence variabilities including the $tRNA^{Pro}$ gene and the left domain of the control region detected in the hatchery strains and wild populations of Japanese flounder

	Hatchery strains			Wild populations		
	HY	HF	HC	HKD	TTR	CHB
Sample size	100	100	100	50	69	71
Number of variable sites	43	29	37	76	87	103
Number of substitutions	42	28	35	81	91	110
Transitions	39	24	33	70	80	95
Transversions	3	4	2	11	11	15
Number of haplotypes	14	4	7	48	65	66
Haplotype diversity (h)[*1]	0.798	0.692	0.793	0.998	0.998	0.998

[*1] Haplotype diversity is given by $h=(1-\Sigma x_i^2)\ n/(n-1)$, where x_i is the frequency of a haplotype and n is the sample size (Nei and Tajima, 1981)

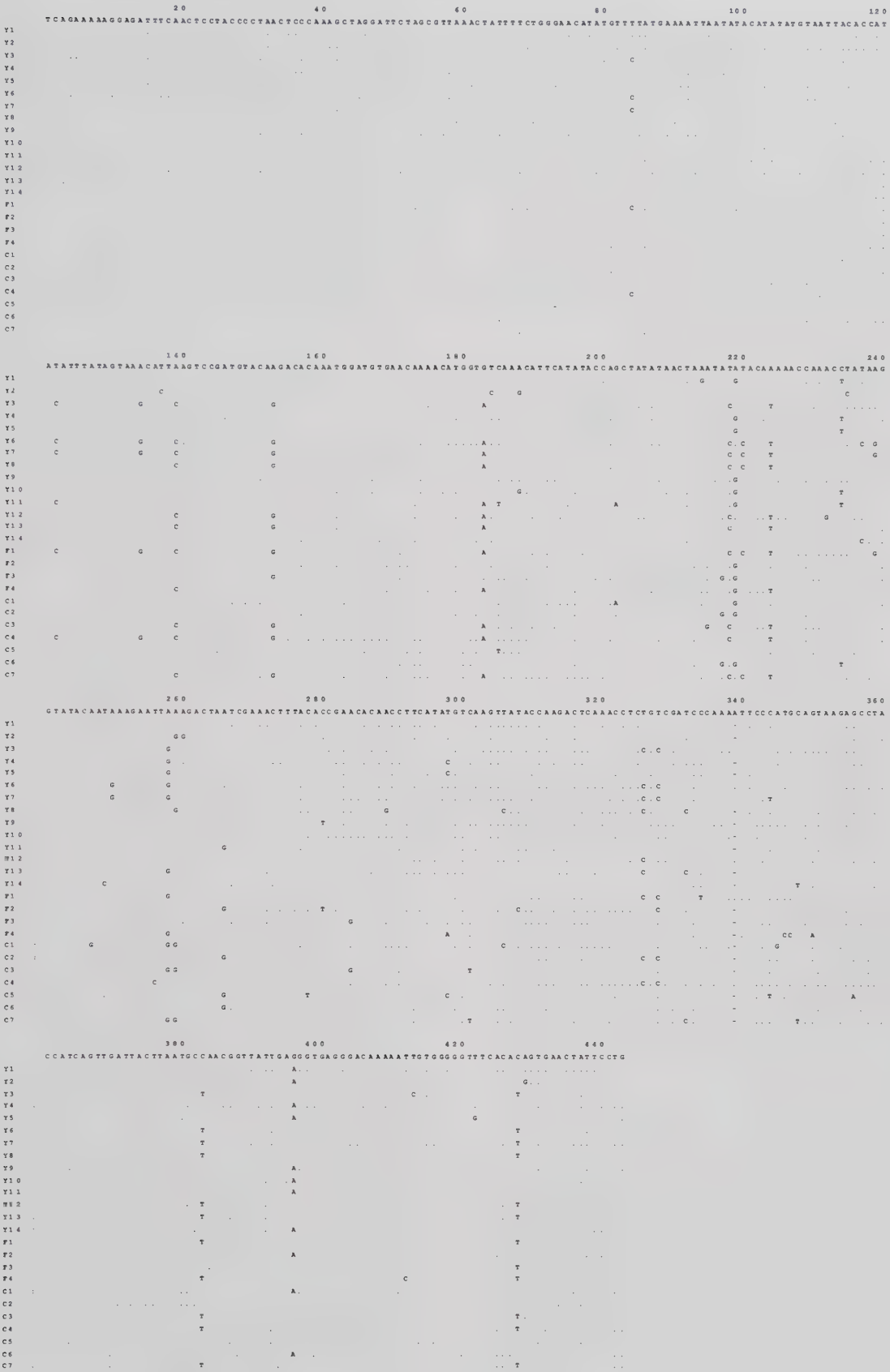

Fig. 5. Sequences of 25 mtDNA-haplotypes identified among the 300 individuals in the 3 hatchery strains (HY strain: 14 haplotypes, Y1-Y14; HF strain: 4 haplotypes, F1-F4; HC strain: 7 haplotypes, C1-C7). Identity with the reference sequence is indicated by dots, and a dash (-) represents a single nucleotide deletion/insertion.

Table 15. Number of mtDNA haplotypes commonly shared between the hatchery strains and wild populations

Haplotypes[*1]	Hatchery strains			Wild populations		
	HY	HF	HC	HKD	TTR	CHB
Y5	1	0	0	0	0	1
Y10	1	0	0	1	0	0
Y11	4	0	0	0	0	1
F1	0	45	0	0	2	0
C3	0	0	12	0	0	1

[*1] Sequence of each haplotype is shown in Fig. 5

Table 16. Estimates of F_{ST} (Φ_{ST}) value based on microsatellites and mtDNA sequences in several combinations of samples

Sample combinations	Microsatellites		mtDNA	
	F_{ST}	p	Φ_{ST}	p
Global (no subdivision)	0.055**	0.000	0.123**	0.000
Among hatchery strains	0.088**	0.000	0.187**	0.000
Among wild populations	0.004*	0.003	0.005	0.134
Hatchery *vs* wild group[*1]	0.019**	0.000	0.046**	0.000
HY *vs* HKD	0.026**	0.000	0.084**	0.000
HF *vs* TTR	0.086**	0.000	0.150**	0.000
HC *vs* CHB	0.034**	0.000	0.079**	0.000

[*1] Allele (mtDNA haplotype) frequency estimated for the 3 hatchery strains and the 3 wild populations were pooled into a hatchery group and a wild group, respectively

The F_{ST} (Φ_{ST}) values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ and **=$p<0.001$

females, it can be concluded that only 25% of the candidate female broodstock for both strains (HY strain: 14/50; HC strain: 7/30) were effective to found each strain, although it was not possible to estimate the number of effective males.

Gene diversity (H_e) associated with the microsatellite loci did not show pronounced differences between the hatchery strains and wild populations, although it is subject to one exception: a significant reduction of the H_e value was observed in the HF strain (see also below). The Kruskal-Wallis rank sum test, which is a conservative and non-parametric analysis, indeed revealed that there was a significant difference of the mean H_e values between the hatchery strains and wild populations, however, a previous genetic assessment of Japanese flounder using four microsatellite loci (Yoshida *et al.*, 2000), and several studies for other fish species (reviewed in O'Connell and Wright, 1997; Coughlan *et al.*, 1998; Norris *et al.*, 1999; Desvignes *et al.*, 2001) demonstrated that allelic diversity was substantially reduced in hatchery strains without significant differences of H_e values between hatchery strains and wild-populations. In so far as a first generation hatchery strain, these results are hardly surprising since an estimate of heterozygosity could be inflated if the strain of interest was founded using heterozygous parents. A population bottleneck of short duration may possibly reduce the number of alleles without significant losses of heterozygous individuals (Allendorf, 1986; Allendorf and Ryman, 1987). While, it is likely that significant reductions of both microsatellite and

mtDNA variabilities detected in the HF strain might be caused by a population bottleneck together with occurrences of inbreeding events when this strain was founded. This is because the HF strain was founded using both wild caught fish and broodstock maintained in this hatchery, although the level of inbreeding, if any, seems not to be high since homozygote excess was not evident in this strain ($H_o/H_e=0.97$), and moreover, the F_{IS} value estimated for this strain was indeed higher compared with other samples, but not significant ($F_{IS}=0.018$, $p=0.20$).

Strain/population differentiation

The high F_{ST} and Φ_{ST} values estimated for between the hatchery strains, and between the hatchery strains and the wild populations, exhibit that there was pronounced genetic differentiation between these samples. This is in contrast to the results obtained by the previous mtDNA-RFLP approach (Sugaya *et al.*, 1999), which revealed that about a half of the 15 haplotypes detected in four Japanese flounder hatchery strains were commonly shared between at least two strains. These authors also reported that genetic heterogeneity between a hatchery group into which four hatchery strains were pooled and a wild group into which four wild populations were pooled was not significant. The results presented here do not support their findings since there was significant evidence of genetic heterogeneities between the hatchery and the wild group (Table 16: $F_{ST}=0.019$, $p=0.000$; $\Phi_{ST}=0.046$, $p=0.000$). The limited types of restriction endonucleases available for the use in the RFLP method (*Hpa* Ⅱ, *Hae* Ⅲ, and *Hind* Ⅲ) may not necessarily be satisfactory to detect genetic differentiation between strains on a fine scale, it is not clear however as regards the foundation and maintenance of the strains for which they screened.

Summary

This chapter demonstrated that the simultaneous use of the 11 microsatellite loci and the sequences of the mtDNA control region is a powerful approach to monitor genetic diversity within and between Japanese flounder hatchery strains. This chapter also revealed that there was pronounced genetic differentiation between the hatchery strains and the wild populations, and the loss of microsatellite alleles and mtDNA haplotypes in hatchery strains was typical due most likely to the limited number of effective parents. It should be noted that further massive stocking of hatchery-reared fish into the natural environment might possibly result in irredeemable losses of alleles (haplotypes) in natural stocks. The only way to minimize genetic impacts of stocking to natural stocks is to improve the genetic management of hatchery broodstock.

Chapter 5. Microsatellite-based pedigree reconstruction of a Japanese flounder hatchery strain

As seen in the preceding chapter, the genetic variability of Japanese flounder hatchery strains to be stocked into the natural environment is typically reduced. Rate of loss of genetic variability in a population is based on the effective population size (Primack, 1998), and the extent of the effective population size is affected by several factors such as a small number of founders, unequal sex ratio in breeding populations, and family-size variations (Gall, 1987; Hedrick, 2000). It is thus recommended that hatchery managers should strive to minimize the effects of these possible causes of reduced effective size, and such factors can be accurately evaluated by an examination of the pedigree structure in the strains of interest.

Almost all of the flounder hatcheries employ the mesocosm spawning method to produce flounder-seedlings available for stocking practices. Using this method however it is difficult to control the mating behavior of the broodstock in the spawning tanks, and thereby it is complex to predict the pedigree structure of reproductive output. In practice, hatchery-reared flounder are usually bred without any

consideration to the mating system, and consequently the pedigree structure of flounder hatchery strains seem to deviate far from the ideal conditions (*e. g.*, equal number of male and female parents, random distribution of family size) (Chapter 4; Fujii, 2001). While, intentional culling practice, usually for larger size in total length, is commonly operated in flounder hatcheries to encourage the survival of released flounder-seedlings in the natural habitat. Culling for particular traits however has the potential to give rise to a large family-lineage bias through selection of siblings (Allendorf and Phelps, 1980; Allendorf and Ryman, 1987), possibly resulting in the reduced effective size of released fish due to a large variance in family size. The pedigree structure therefore should ideally be monitored in both breeding and release programs as a routine hatchery operation in order to improve flounder hatchery management.

The microsatellite profiling technique has been acknowledged as an efficient approach to examine pedigree structure in mixed family tanks of several aquatic organisms (Herbinger *et al.*, 1995; O'Reilly *et al.*, 1998; Herbinger *et al.*, 1999; Perez-Enriquez *et al.*, 1999; Norris *et al.*, 2000; Huvet *et al.*, 2001; Selvamani *et al.*, 2001). This chapter illustrates an example of microsatellite-based pedigree reconstruction in a Japanese flounder hatchery strain, and describes several implications for further hatchery management options. The possible impacts of the stocking practices to the wild populations are also discussed. The number of broodstock parents used was relatively small (18 wild captives), however, the hatchery procedures by which the strain was created is essentially the same to those operated in other flounder hatcheries.

Materials and methods

Samples of reproductive output

Hatchery production was initiated in May 2001 at the Miyako Hatchery Station of the Japan Sea-Farming Association. A total of 18 fish including 12 ripe females (FM#1-#12) and six ripe males (M#1-#6), which were wild captives caught in Miyako Bay (142°E, 40°N), were allowed to spawn in a spawning tank. Sexual maturity and health conditions of these fish were examined through the hatchery operations prior to the mating event, and all fish were regarded to be in suitable condition for reproduction. Approximately one million eggs were produced during one night (24 May) without the aid of artificial stripping, and the eggs were transported to a 0.5 ton incubation tank. The hatching success of eggs was estimated by counting the number of eggs and larvae per unit volume (volume cubic method), and it turned out that almost all the collected eggs successfully hatched out (*i. e.*, approximately 100% success of fertilization and hatching). Out of which 600,000 F_1 offspring were randomly collected and communally reared in a 50 ton tank for four months. At four months of the age, the offspring were size-sorted for larger size in total length through a routine hatchery operation, and then the selected fish were tagged and stocked into Miyako Bay (60,000 fish).

Samples of offspring for pedigree reconstruction were collected at three stages during the rearing period. An arbitrary sample of 113 larvae was collected within 24 hours after hatching (sample abbreviation: OP0), and 216 fish were randomly collected at the age of one month (abbr., OP1). A further 407 fish were sampled at four months of the age (abbr., OP4) which was timed with the stocking practice, and this sample was size-sorted (see above): just before the stocking event, 207 individuals were sampled from the fish that were selected for the release (total length, 53.8-97.2mm; mean$\pm$s.d. 78.0$\pm$7.3mm), and 200 individuals were sampled from the fish selected against (*i. e.*, not to be released) (total length, 37.2-59.4mm; mean$\pm$s.d. 50.1$\pm$3.7mm). These subsets of non-arbitrary fish were sampled to address the question of whether routine culling operations (size-dependent) in hatcheries would generate a significant skew of family size between the

released and non-released fish.

Parentage determination and statistical analysis

Four microsatellites (*Po1, Po13, Po42,* and *Po91*; Chapter 2) were profiled for all fish samples. Once the microsatellite genotypes of all candidate broodstock and offspring were profiled, each offspring was assigned to its parents by looking at whether the potential parental couples would exactly generate the offspring's genotypes for all loci.

The contribution of each family to the released fish was evaluated using the size-sorted sample (OP4), as the ratio of the number of released fish divided by the number of released fish plus non-released fish. The estimated value was compared with the expectation in each family (released fish: non-released fish=1:1) by using the *G*-test for goodness of fit (Sokal and Rohlf, 1997) with the Williams's correction by which the *G* value can be approximated better to the chi-square distribution (Williams, 1976). The significance level for all multiple tests was adjusted according to the method described by Rice (1989).

The effective population size was calculated based on the formula described by Lande and Barrowclough (1987). First, the effective number of female parents (N_{ef}) and male parents (N_{em}) were calculated according to the formula: $N_{ef}=(N_f k_f-1)/(k_f-1+V_{kf}/k_f)$ and $N_e m=(N_m k_m-1)/(k_m-1+V_{km}/k_m)$, where N_f and N_m is the census number of female and male parents, k_f and k_m is the mean number of offspring per female and male, and V_{kf} and V_{km} represents the variance in family size for each sex, respectively. This approach assumes non-Poisson distributions of gametes for each sex. The N_{ef} and $N_e m$ value were then combined to calculate the overall effective size (N_e) as follows: $N_e=4N_{ef}N_e m/(N_{ef}+N_e m)$.

Possible stocking impact on the effective size maintained in a wild population (N_w) was approximated on the basis of the formula: $1/N_{et}=x^2/N_c+(1-x)^2/N_w$, where N_{et} is the total effective size of the wild population and released hatchery fish, N_c is the effective size of the hatchery fish, and x is the relative contribution from the hatchery fish (Ryman and Laikre, 1991). The effective size of 7,900, which was estimated for a wild population described in Chapter 3 (IWT population), was used as the N_w value.

Results

Allelic variations of the four loci proved to be extensive in 18 potential broodstock (Table 17), and the number of alleles varied depending on the locus (minimum 13 at the *Po42* locus, maximum 19 at the *Po91* locus). Unique alleles, that is, alleles unique to just one individual, were detected for all loci. At the three loci (*Po1, Po13,* and *Po91*), the number of unique alleles was more than a half of the total number of detected alleles. That is, 15 of the 18 broodstock had unique alleles for at least one locus. These unique alleles were efficient indicators to trace pedigrees.

The parentage determination successfully assigned all the offspring back to a single parental couple. Table 18 shows the family size of each family observed in each of the three samples (OP0, OP1, and OP4 samples). A total of nine families were identified across the three samples, and there was an apparent difference of family size among the families. Six families were attributable to one male (M#2): more than 99% offspring were sired by male M#2, and two other males (M#1 and #6) sired only a very few offspring. Three males (M#3, #4, and #5) and six females (FM#1, #2, #3, #5, #6, and #8) did not contribute to the next generation.

A significant difference of the proportion of family size between samples was observed in three families after correction of the significance level for 18 simultaneous test ($p<0.0028$), in family E (between OP0 and OP1 samples, $z=3.68$, $p=0.0002$; OP0-OP4, $z=3.46$, $p=0.0005$), family G (OP0-OP1, $z=4.13$, $p=0.0000$), and family I (OP1-OP4, $z=3.03$, $p=0.0024$, marginal significance). Although the causes of the significant fluctuations in family size can not evidently be determined, these results might be

due to differences in the initial family survival performance. Given the fact that all families identified in the OP0 sample (0 day offspring) appeared in both OP1 (1 month) and OP4 (4 months) samples, the differences of family survival performance, if any, did not result in the demise of any particular family.

The *G*-test revealed that the contribution of offspring to the released fish significantly departed from the expectations in three families, family E, F, and G (Table 18). In families E and G, the contribution to the released fish was significantly lower than that to the non-released fish (family E, $G_{adj}=8.26$, $1df$, $p<0.008$ after correction for 6 simultaneous tests; family G, $G_{adj}=17.55$, $1df$, $p<0.008$, 6 simultaneous tests), while the contribution to the released fish in the family F was significantly high ($G_{adj}=38.19$, $1df$, $p<0.008$, 6 simultaneous tests), suggesting that culling operations would have the potential to select for particular family lineages. The remaining three families, family D, H, and I, did not show any significant deviations from the expectations ($1df$, $p>0.008$, 6 simultaneous tests).

The effective size of this strain was estimated to be of 3.3 (average of the 3 samples) after correction of unequal sex ratio coupled with unequal family size, and it was decreased by 80% of that calculated assuming an ideal situation, that is, all the potential broodstock could equally contribute to the offspring pool ($N_e=16$). Total effective size (N_{et}: wild population and hatchery strain) was also estimated to see the extent of the stocking effect to the total effective size (Fig. 6). The N_{et} value was drastically decreased even though the relative contribution from the hatchery strain was minimal: 20% contribution from the hatchery strain was sufficient to diminish 99% of the ini-

Table 17. Genotypes of 4 microsatellites determined for the 18 candidate broodstock

Candidate broodstock		Genotypes			
Males	TL(cm)*1	***Po1***	***Po13***	***Po42***	***Po91***
M#1	50.0	162/196	208/214	172/174	148/156
M#2	49.5	162/180	224/234*	174/236*	156/158
M#3	43.6	198*/222*	212/228*	178/182	160/160
M#4	42.7	164/188	208/210*	178/194*	160/170*
M#5	38.2	162/164	218/232	172/192*	150/160
M#6	45.9	162/162	214/216	172/182	162/166*
Females					
FM#1	53.2	164/208*	232/238*	172/178	160/222*
FM#2	49.5	162/186*	216/236*	172/178	156/182*
FM#3	54.6	162/162	212/216	182/190	162/198*
FM#4	53.8	180/206*	208/212	172/180	190*/194
FM#5	51.0	184/188	208/216	172/176	148/148
FM#6	50.0	162/178*	216/260	172/172	150/194
FM#7	51.7	162/184	216/242*	180/196*	160/176
FM#8	50.5	190/204*	220*/250*	190/190	162/164*
FM#9	54.3	164/196	208/218	176/202*	150/178*
FM#10	50.0	162/162	208/212	176/180	156/156
FM#11	48.0	190/194*	208/212	186/186	146*/184*
FM#12	48.0	162/188	216/224	178/178	154*/158
No. of alleles		15	16	13	19
No. of unique alleles		8	9	5	11

Unique alleles, alleles unique to one individual, are denoted on the individual genotypes by adding an asterisk
*1 Total length

tial effective size of the wild population (N_{et} =83). It is generally recommended that an effective size of 50 should be maintained in a short-term hatchery production, and 500 be desirable for a long-term self-sustainable hatchery production (FAO/UNEP, 1981). As seen in Fig. 6, the reference figures (N_e=50 and 500) indeed slowed the rate of loss of total effective size, however, a considerable reduction of the initial effective size of the wild population could be occurred as the increase of the contribution from the hatchery strain. The effective size of 500 is also considered as a minimum viable population size (MVP) in the context of genetic conservation (Franklin, 1980; Lande, 1988). The MVP value could be maintained when the contribution from the hatchery strain with N_e=3.3 is of less than 10%, and less than a 30% contribution from the hatchery strain with N_e=50 could maintain the total effective size above the MVP value.

Discussion

In most hatchery strains, an unequal contribution of broodstock to the next generation seems to be typical, particularly in cases that the mesocosm spawning method is employed (Taniguchi *et al.*, 1983; Sugama *et al.*, 1988; Perez-Enriquez *et al.*, 1999; Fujii, 2001). The hatchery strain screened in this study is an extreme case, especially regarding the broodstock males: more than 99% of the offspring turned out to have been sired by a single male (M#2) (Table 18). This is somewhat surprising given empirical observations of the mating behavior in Japanese flounder: one female is followed by several males before the release of eggs, and then she mates with multiple males simultaneously. Possible factors leading to the unsuccessful reproduction of other males, such as a poor sperm quality and gametic incompatibility, are very unlikely since the hatching success of the eggs was approximately 100%. There are no records available regarding the mating behavior in the spawning tank in which the strain was founded; a scenario can be drawn such that this male simultaneously (or alternately) mated with multiple females, and there might have been severe reproductive competition among

Table 18. Parental couples of the offspring determined on the basis of the microsatellite profiles, and the number of offspring in each family (family size)[*1]

Families		A	B	C	D	E	F	G	H	I
Male parents		M#1	M#1	M#6	M#2	M#2	M#2	M#2	M#2	M#2
Female parents		FM#4	FM#11	FM#11	FM#4	FM#7	FM#9	FM#10	FM#11	FM#12
Offspring samples	(*N*)									
OP0[*2]	113	0(0.0)	0(0.0)	0(0.0)	29(25.7)	14(12.4)	33(29.2)	22(19.5)	4(3.5)	11(9.7)
OP1[*3]	216	1(0.5)	0(0.0)	0(0.0)	39(18.1)	44(20.4)	41(19.0)	68(31.4)	13(6.0)	10(4.6)
OP4[*4]	407	0(0.0)	1(0.2)	1(0.2)	79(19.4)	71(17.4)	100(24.6)	105(25.9)	18(4.4)	32(7.9)
#Released	207	0(0.0)	1(0.5)	0(0.0)	48(23.2)	24(11.6)	78(37.6)	31(15.0)	7(3.4)	18(8.7)
#Non-released	200	0(0.0)	0(0.0)	1(0.5)	31(15.5)	47(23.5)	22(11.0)	74(37.0)	11(5.5)	14(7.0)
Percent of each family in the released group		NA	NA	NA	60.8	33.8	78.0	29.8	38.9	56.3
G_{adj} value(1*df*)[*5]		NA	NA	NA	3.8	8.3*	38.2*	17.6*	1.5	0.5
p		-	-	-	0.051	0.004	0.000	0.000	0.215	0.475

[*1] Proportion of the family size in each sample is shown in parenthesis(%)
[*2] Random sample collected within 24 hours after hatching
[*3] Random sample collected at 1 month after hatching
[*4] Size-sorted sample collected at the age of 4 months (For details, see text)
[*5] Significance was tested by using the *G*-test(Sokal and Rohlf, 1997) with the Williams's correction (Williams, 1976). Null hypothesis was assumed as the ratio of the released fish and non-released fish was of 1: 1. Significance was denoted by adding *=$p<0.008$ after correction for 6 simultaneous tests. The *p* value is also shown below the G_{adj} value. For family A, B, and C, the *G*-test was not conducted(NA).

males at the mating event.

The effective size of this strain was of just 3.3, and taken this situation, 15-fold or larger number of founders and spawning tanks needed to be prepared to achieve the effective population size of 50, which is a reference figure generally recommended for a short-term hatchery production related to stock enhancement programs (FAO/UNEP, 1981). It is evident that the successful contribution of this strain to the natural environment will result in a highly destructive impact to the total effective size (Fig. 6), and the recommended figure for short-term hatchery production (N_e=50) do not promise to overcome the risk of significant loss of the total effective size; nevertheless hatchery managers should strive to reach the guideline to relieve the effects of the potential stocking impact as much as possible.

One of the most effective hatchery options to increase the effective size in hatchery strains would be to employ the stripping method with one-to-one crossing using a large number of broodstock, which is expected to lead to more homogenous contribution of the broodstock parents. The stripping method is however difficult to practically adopt in routine flounder hatchery procedures due to several constraints such as the nocturnal spawning behavior and the handling-vulnerability of this species. Therefore hatchery management should be improved so as to achieve a large effective size on the premise that the hatchery production will continue to rely on the use of the mesocosm spawning method. The strain screened in this study originated from the fertilized eggs produced in a single night with a relatively small number of candidate broodstock, and thus it can not be set aside the possibility that other potential broodstock would produce offspring in the other days; Hirano and Yamamoto (1992) reported that ripe females released eggs intermittently but irregularly during the spawning period that lasts for as long as three months (individual spawning days per spawning period was estimated to be of 66-88% in the five females they examined), although there have been limited data available as to the reproductive rhythm of males. In such a case, temporal collections of the fertilized eggs produced by variable-in-time parental couples could encourage the increase of the effective size. Alternatively, assuming that the skewed contribution of broodstock was largely caused by an interaction which arose among the potential broodstock such as a reproductive competition (see above), the effective size could be increased by subdividing the broodstock into multiple spawning tanks to minimize the effects of detrimental interactions.

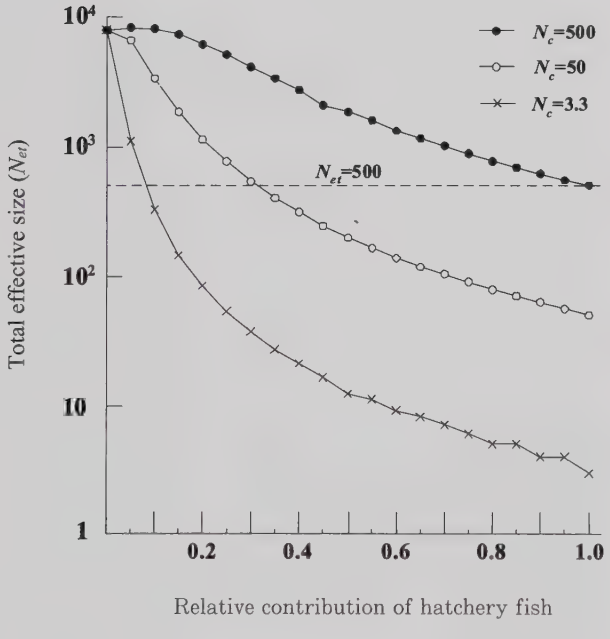

Fig. 6. Total effective size (N_{et}) of wild and hatchery (released) fish (Ryman and Laikre, 1991). The x-axis indicates the relative contribution of released hatchery fish to the total fish (wild plus hatchery fish). The y-axis (N_{et}) is scaled in common logarithm. N_c represents the effective size of hatchery fish, and the initial effective size of wild fish is assumed as of 7,900 (see text). The minimum viable population size of 500 (Franklin, 1980; Lande, 1988) is shown by dotted line.

Another important finding in this study is that the culling operation for larger size fish generated significant deviations of family size between the released and non-released fish. The noticeable variance in the early growth of offspring might be related to the family growth performance. It should be emphasized that even

if an idealized strain with a large number of founders is initially created, culling operations might cause a large family size variance of released fish through selection in favor of a few family lineages. The present author therefore suggests that culling operations for particular traits be avoided to minimize the risk of unexpected pedigree structure to be a possible cause of the reduced effective size.

Summary

This chapter demonstrated that the microsatellite profiling technique would provide the most efficient means to trace pedigrees in mixed family tanks of Japanese flounder. The contribution of broodstock parents to the next generation in the strain screened in this study highly skewed, and such a skewness would be frequently seen in every hatchery production. The present author thus suggests that monitoring of pedigree structure ideally be implemented in every case of hatchery productions. At the same time, the hatchery management options recommended above should be adopted to encourage the contribution of broodstock parents as much as possible.

Chapter 6. Management strategy to minimize loss of genetic variations in closed hatchery strains

It is generally recommended that a large number of unrelated wild fish should ideally be used as the founders of hatchery strains to be reintroduced to the natural habitat so that the genetic variability can be maximized, and at the same time, that the genetic impacts upon the wild fish populations can be minimized (FAO/UNEP, 1981; FAO, 1993). Such recommendations may sometimes be difficult to adopt in practical hatchery-production procedures due to several constraints involving the labor and budget intensity associated with the collection and maintenance of wild fish, and thereby hatchery managers may be constrained to use a subset of hatchery-based fish with a few wild fish as founders to create the descendent generations. This surely enhances the decay of genetic variability in the subsequent generations through a severe genetic drift coupled with inbreeding. In such cases, it is crucial to explore ways that the further loss of founders' genetic variations can be minimized.

Maximal retention of genetic variations from captive populations to the descendants could be achieved through the selection and mating of genetically important individuals in the focused populations, rather than through random mating (Ballou and Lacy, 1995). Several measures to evaluate the genetic importance of individuals have been proposed such as the founder importance coefficient (*fic*: Ballou and Lacy, 1995), genome uniqueness (*gu*: MacCluer *et al.*, 1986), and mean kinships (*mk*: Ballou and Lacy, 1995). A computer simulation study conducted by Ballou and Lacy (1995) suggested that the *mk* provides the most efficient measure to rank genetically important individuals. The *mk* measure is calculated based on the kinship coefficient (f_{ij}: Falconer and MacKay, 1996), which is the probability that alleles possessed by two individuals at the same locus are identical by descent. The mean kinship of individual i is defined as the average f_{ij} value between individual i and all other individuals including itself, and the individuals with low *mk* values are ranked as genetically important individuals (Ballou and Lacy, 1995). The breeding system using the *mk* estimator (mean kinship approach, Ballou and Lacy, 1995) is proceeded as the lowest-ranked male is mated with the lowest-ranked female, and after removing these lowest-ranked individuals for both sexes, the *mk* value is then re-calculated for all the possible combinations of the remaining individuals, and again the individuals with the lowest rank are paired. This procedure is continued until the required number of offspring is obtained depending on the husbandry's capacity. This strategy, in other words, gives a high priority to the individuals with the lowest degree of relatedness to other members in the population, whose alleles have a risk to be undelivered to

the next generations (Rodriguez-Clark, 1999; Lacy, 2000).

It appears that the breeding strategy based on the mean kinship values, which is conceptually called as minimal kinship selection (Doyle *et al.*, 2001), can serve as an efficient hatchery management option to slow the loss of genetic variations in captive populations (Ballou and Lacy, 1995; Montgomery *et al.*, 1997; Rodriguez-Clark, 1999; but see Caballero and Toro, 2000; Fernandez and Caballero, 2001), the practical application of the minimal kinship selection (*mk* selection) however needs the knowledge of the genealogies to estimate the f_{ij} value, which is not available in most cases in Japanese flounder hatchery strains. An alternative measure to clarify the relatedness between individuals is thus required to apply the *mk* selection approach to non-pedigreed strains. A currently available statistic to estimate inter-individual relatedness without pedigree information is the pairwise relatedness coefficient r_{xy}, devised by Queller and Goodnight (1989), as the availability to classify individuals by kinships has extensively been documented (*e. g.*, Blouin *et al.*, 1996; Taylor *et al.*, 1997; Fontaine and Dodson, 1999; Norris *et al.*, 2000).

The first purpose in this chapter is to validate the microsatellite-based r_{xy} statistic as a kinship estimator in a Japanese flounder hatchery strain. Eleven microsatellite markers for which the previous chapters demonstrated the utility were employed to calculate the r_{xy} statistic. Secondly, the present author discusses the potential of the *mk* selection approach in preservation of the genetic variations in Japanese flounder hatchery strains, and several management options applicable to the hatchery production are proposed. In this study, a hatchery strain of Japanese flounder founded by wild captives is referred to a source strain for which the generation is denoted as G_1, and the founder generation (wild captives) is regared as G_0. The focus of interest in this chapter is to explore an effective way to retard the loss of the genetic variability from the generation G_1 to the next generation with a few or no available genetic inputs.

Materials and Methods

Pedigree reconstruction

A hatchery strain was created at the Miyako Hatchery Station by placing a total of 14 candidate parents (G_0 fish) including six ripe females (F#1-#6) and eight ripe males (M#1-#8) in a spawning tank for four days. Approximately 500,000 larvae were produced, of which 83 fish sampled at the age of one month (G_1 offspring) together with the 14 captive broodstock parents were screened for the 11 microsatellites (*Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48, Po52, Po56*, and *Po91*, see Chapter 2), and the pedigrees were reconstructed in the same manner described in the preceding chapter. The pedigree information was further used to validate the r_{xy} statistic (Queller and Goodnight, 1989) as a kinship-estimator (see below).

Estimation of pairwise relatedness coefficient

The pairwise r_{xy} statistic, which is calculated correcting a bias for the population allele frequencies by which the relatedness value between individuals x and y will possibly be biased (Queller and Goodnight, 1989), was estimated for all possible dyads within the G_1 offspring based on the individual microsatellite genotypes. The r_{xy} values obtained were compared among three categories including full sibs, half sibs, and unrelated pairs by means of the Kruskal-Wallis nonparametric analysis (Sokal and Rohlf, 1997) using the statistical analysis software STATPARTNER version 4.5 (O-ha Inc., Tokyo). Misclassification rate of the r_{xy} estimator, which is the rate of the errors that an individual in a kinship category is falsely assigned to the other categories, was estimated on the basis of the cut-off value defined as the midpoint between the mean r_{xy} values of any two of the three categories (see Blouin *et al* ., 1996). The utility of the r_{xy} statistic to discriminate the kinships between the G_1 offspring, without the pedigree information, was also addressed by estimating inter-individual

genetic distance, which was simply defined in this study as $D_{(r)}=1.0-r_{xy}$. Negative r_{xy} values were tallied as $r_{xy}=0.0$ (*i. e.*, $D_{(r)}=1.0$). Based on the $D_{(r)}$ matrix obtained, an UPGMA tree topology was constructed using the PHYLIP version 3.6 software package (Felsenstein, 2000), and the tree was visualized by using the TREEEXPLORER software package version 2.12 (provided by Dr. K. Tamura, Tokyo Metropolitan University, Hachioji, Tokyo).

Minimal kinship selection

The average of r_{xy} values between one individual and all the other individuals (mean relatedness: mr_{xy}), was calculated for all G_1 offspring. The original *mk* selection approach devised by Ballou and Lacy (1995) is proceeded by mating between the lowest *mk* male and female by one-to-one crossing (see above), this approach however was not possible to adopt in this study without modifications since there were no records available to determine the sex of the G_1 offspring, and thus the *mk* selection procedures were further simplified, as proposed by Doyle *et al.* (2001), as a subset of lower mr_{xy}-scored fish were selected without doing the recalculation (mk_r selection). It was considered, by the present author, that the mk_r selection would conversely be more applicable to the currently operated production-regimen in Japanese flounder hatcheries as this approach will be more applicable to the mesocosm breeding method than is the original (one-to-one crossing).

The effectiveness of the mk_r selection approach was also examined for a hypothetical situation that the G_1 offspring were kept together with some wild captives. In such a case, it was expected that the wild fish could preferably be selected through the mk_r selection procedures since the wild fish were likely to be unrelated from any members of the G_1 offspring, and since the mk_r selection would theoretically be favorable to the individuals who carry rare genotypes (Ballou and Lacy, 1995). The mk_r selection was applied to a hypothetical strain (abbreviation, G_1^+) consisting of the 83 G_1 offspring and nine wild fish that were collected at Miyako Bay (*i. e.*, the wild fish comprised 10% of this hypothetical strain).

The number of fish to be selected was set as eight, just as same as the census number of the actual G_0 founders (see below). The genetic variability in terms of the allelic diversity (number of alleles per locus: A) and the gene diversity (unbiased expected heterozygosity: H_e) estimated for the mk_r-selected fish was compared with that of randomly generated subgroups from the G_1 offspring (or G_1^+ sample) through the Monte Carlo procedures with 100 iterations.

Computer programs to execute the mk_r selection (including the r_{xy} statistic calculation) and Monte Calro simulation were written by the Visual Basic, and the programs were run on the Excel 2000 software (Microsoft Co., Redmond, WA).

Results

Relatedness estimator r_{xy} to distinguish kinships

The pedigrees of the G_1 offspring were unambiguously determined (Table 19): two of the six females (F#5, #6) and four of the eight males did not contribute to the G_1 offspring pool, and the remaining four females and four males produced 10 pedigrees. A total of 3403 dyads in the G_1 offspring were obtained, of which 513 dyads were categorized as full-sibs, 1085 were as half-sibs, and 1805 were as unrelated pairs. The pairwise r_{xy} values in the G_1 offspring ranged from -0.551 to 0.782 (mean$\pm$s. d., -0.013 ± 0.249). Percentage distribution of the r_{xy} values calculated for all dyads in each kinship category is plotted in Fig. 7. The mean r_{xy} values within the categories were estimated as follows: full-sibs: 0.369 ± 0.173; half-sibs: 0.081 ± 0.166; unrelated pairs: -0.178 ± 0.134. The Kruskal-Wallis rank-sum analysis revealed that the difference of the r_{xy} values was highly significant among and between the categories (overall: $H=2022.9$, $2df$, $p=0.000$; between full-sibs and half-sibs: $H=616.0$, $1df$, $p=0.000$; full-sibs and unrelated pairs: $H=1156.9$, $1df$, $p=0.000$; half-sibs and unrelated pairs: $H=1208.0$, $1df$, $p=0.000$).

When the threshold values (cut-off value) to

classify kinships between individuals described above was applied (Table 20), full-sibs and unrelated pairs were distinguishable with a high accuracy at the cut-off value of $r_{xy}=0.095$, as just 0.2% full-sibs were misallocated into unrelated category, and 3 % unrelated pairs were erroneously classified as full-sibs. Cut-off value defined for between full-sibs and half-sibs ($r_{xy}=0.225$) yielded approximately 20% misclassification rates reciprocally, and 17% half-sibs and 23% unrelated pairs were misclassified into each alternate category at the cut-off value of $r_{xy}=-0.049$. These misclassification rates were close to those estimated by Blouin *et al.* (1996), who studied the potential of the r_{xy} statistic to discriminate kinships, based on 20 microsatellite markers for a set of simulated genealogical lines of house mouse (misclassification rates between full-sibs and unrelated pairs: 2.5%; full-sibs and half-sibs: 15-16%; half-sibs and unrelated pairs: 15-17%).

The UPGMA tree topology showing the sibships of the G_1 offspring reconstructed on the basis of the r_{xy}-based genetic distance is given in Fig. 8 . The tree topology advocated the validity of the distance measure to portray the genealogies: 96% of fish were clustered well in accordance with the true kinships, although there were a few exceptions such that two fish in family B (OP3 and OP69) were more closely clustered with four fish in family F rather than are with the other members in family B, and one of the two fish in family D (OP22) was positioned in the neighborhood of OP42 (family J) apart from the full sibling (OP5).

Minimal kinship selection

The mr_{xy} values of the G_1 offspring ranged from -0.103 to 0.118 (mean$\pm$s.d., -0.013 ± 0.045), and the mk_r selection procedures retrieved the eight lowest mr_{xy} fish (mk-G_1 sample) including two fish from each of the three families (family A, D, and H) and one from each of the two families (family B and E). None of the members in five families (family C, F, G, I, and J) were found in the mk-G_1 sample. Regarding the G_1^+ hypothetical strain consisting of the 83 G_1 offspring and nine wild fish, the mr_{xy} values ranged from -0.189 to 0.125 (mean$\pm$s.d., -0.011 ± 0.052), and eight fish including six wild fish and two G_1 offspring (family D and E) were drawn through the mk_r selection (mk-G_1^+ sample). Table 21 gives the summary statistics of the genetic variability estimated for the two mk_r samples together with that of randomly selected subgroups (G_1-*rand* derived from the G_1 and G_1^+-*rand* from the G_1^+). The comparison between the mk-G_1 sample and G_1-*rand* subgroups showed that the mk_r selection gave a slight benefit in the retention of the allelic diversity (A) than the random selection (mk-G_1: $A=6.18$; G_1-*rand*: mean$\pm$s. d., $A=5.85\pm0.39$, average of 100 iterations). The advantage of this approach to recover the allelic diversity was further strengthened when the mk-G_1^+ and G_1^+-*rand* samples were compared, as the A value was of 7.18 for the mk-G_1^+, and of 6.03 ± 0.57 (mean$\pm$ s. d.) for the G_1^+-*rand*. The A value estimated for the mk-G_1^+ sample was consistently greater than that of the 100 iterated random samples with just one exception: the highest A value ($A=7.36$) was obtained at one instance in the 100 iterations conducted.

The mk_r selection was also effective to recover the gene diversity (H_e) as the mk-G_1 and mk-G_1^+ samples yielded a higher H_e value (mk-G_1: $H_e=0.78$; mk-G_1^+: $H_e=0.79$) than the average of the arbitrary subgroups (G_1-*rand*: mean$\pm$s. d, 0.76 ± 0.02; G_1^+-*rand*: 0.77 ± 0.03). It should however be stressed that heterozygosity would be insensitive to a population bottleneck for a short duration, and not greatly be affected by the loss of low frequency alleles (Chapter 4; Allendorf, 1986). This can be seen in the results presented here: both the mk_r-selected samples (mk-G_1 and mk-G_1^+) and arbitrary subgroups (G_1-*rand* and G_1^+-*rand*) generated a comparably higher level of gene diversity than the source strain (G_1 offspring: $H_e=0.69$; G_1^+ sample: $H_e=0.70$), despite the fact that the allelic diversity maintained in the source strains were lost in the selected subgroups, suggesting that the gene diversity alone not be used as a measure of the genetic variability in captive populations.

Table 19. Contribution of the G_0 broodstock parents to the G_1 offspring pool

Families	A	B	C	D	E	F	G	H	I	J	
Parents											
Males	M#2	M#6	M#8	M#2	M#5	M#6	M#5	M#6	M#5	M#2	
Females	F#1	F#1	F#1	F#3	F#3	F#3	F#4	F#4	F#2	F#2	Total
Number of offspring	16	10	12	2	21	4	11	5	1	1	83

Table 20. Possible errors to misclassify individuals by relatedness estimator (r_{xy}) and the misclassification rates estimated for between the kinship categories

Type of errors		Cut-off value* (r_{xy})	Misclassification rate (%)
True kinship	Misclassified as #		
Full-sibs	Half-sibs	0.225	20.7
	Unrelated	0.095	0.2
Half-sibs	Full-sibs	0.225	19.8
	Unrelated	−0.049	22.8
Unrelated	Full-sibs	0.095	2.8
	Half-sibs	−0.049	16.8

* Cut-off value was defined as the midpoint value between any two categories (Blouin *et al.*, 1996)

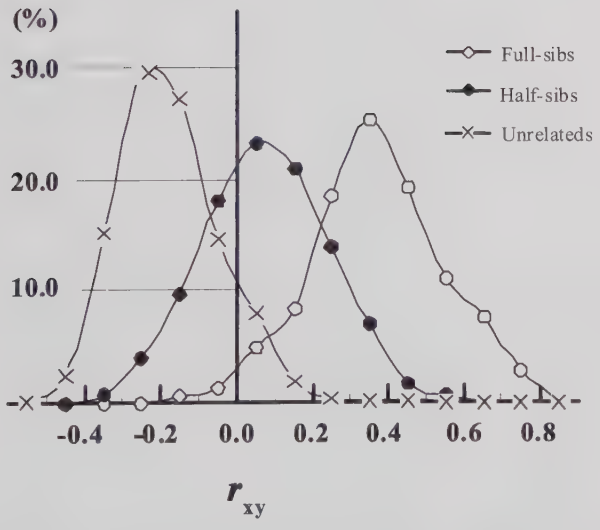

Fig. 7. Percentage distribution of the relatedness estimator estimated r_{xy} for full-sib, half-sib, and unrelated categories in the G_1 offspring. The number of dyads in each category is as follows: full-sibs: 513 dyads; half-sibs: 1,085 dyads; unrelated pairs: 1,805 dyads.

Discussion

Relatedness estimator to resolve kinships

As in a common hatchery-production practice of the Japanese flounder, reproductive outputs are kept and reared in mixed family tanks without any external tags, and unfortunately, it is usual that the historical records of the established strains are not available, and therefore pedigree tracing is difficult, or rather, impossible. This is of a special concern when further generations are constrained to be created by using such non-pedigreed strains, of which there might be highly related individuals. Sib mating should be avoided to minimize the rate of inbreeding in order to counter the threat of inbreeding depression, which may possibly be embodied within a few generations (Princee, 1995 and references therein). Inference of sibships for non-pedigreed captive (or wild) populations in the absence of the pedigree information has been a focus of recent attention

Fig. 8. UPGMA tree topology showing the sibships between individuals in the G_1 offspring on the basis of the genetic distance, $D_{(r)}=1-r_{xy}$ (see text). The tree is rooted by the midpoint rooting method. Labels at branch tips indicate the identity number of offspring, and the pedigree of each offspring is also given in parenthesis.

(Ritland, 2000); the relatedness estimator r_{xy} based on the 11 microsatellite markers employed in this study proved to be useful as a measure of the identity by descent, as the use of the midpoint cut-off value achieved to discriminate the fish in full-sib category from unrelated pairs, or *vice versa*, with few misclassification errors, although there was a relatively high probability of misclassification errors arising between the half-sib and the other categories (approximately 20% misclassification rates). The r_{xy} statistic sometimes entails a wide range of variations even within a kinship category as Queller and Goodnight (1989) pointed out; nevertheless the present author suggests that the microsatellite-based r_{xy} statistic provides an efficient tool as a kinship indicator with the practical uses to minimize the risk of sib mating. The ability of the r_{xy} statistic to resolve kinships was also verified by the attempted tree-topology reconstruction given in Fig. 8. This graphical analysis would be helpful to give a brief overview of sibships between individuals in a non-pedigreed hatchery strain.

Minimal kinship selection

The minimal kinship selection strategy (*mk* selection) following a proposal by Ballou and Lacy (1995) is widely approved as the most efficient and intuitively understandable approach to recover the genetic variations in a captive population. The mk_r selection, which is the simplified version of the *mk* approach (Doyle *et al.*, 2001) employed in this study, also had a benefit to retain the genetic variability compared with the random selection. Given the theory that the *mk* concept will give a focus on the individuals with rare genotypes (Ballou and Lacy, 1995), the efficiency of the *mk* selection in allelic retention would depend on the frequency of rare alleles maintained in the population of interest. As seen in this study, adding a few exotic alleles (wild fish) to the G_1 offspring that had a relatively low level of allelic variations due to the limited number of founders (4 females and 4 males) gave a better performance of the mk_r selection. While, it should be noted that none of the members in several families were found in the mk_r selected subgroup. These results are, however, barely surprising since the *mk* concept aims to find out the genetically important individuals whose alleles are not common in the studied population with the notion that underrepresented founder alleles should be propagated, by which genetic variations could be ensured (Ballou and Lacy, 1995; Lacy, 1995).

Although the high utility of the *mk* selection strategy for the management of captive populations has been accepted in the context of conservation genetics as cited above, there seems to be still room for a measure of disagreement about the *mk* concept: this approach has a drawback upon reducing the rate of inbreeding (Caballero and Toro, 2000). A previous simulation analysis studied by Fernandez and Caballero (2001) concluded that the *mk* selection approach applied to hypothetical populations indeed offered a

Table 21. Genetic variability in terms of the allelic diversity (number of alleles per locus: A) and gene diversity (unbiased expected heterozygosity: H_e; Nei, 1987)

	Parental generation (G_0)*1		G_1 offspring			G_1 offspring plus wild fish (G_1^+)*2		
	Candidates	Actual parents	Overall	*mk*-G_1	G_1-*rand*	Overall	*mk*-G_1^+	G_1^+-*rand*
Number of fish	14	8	83	8	8	92	8	8
Allelic diversity(A)	10.64	8.27	8.18	6.18	5.85(±0.39)	10.73	7.18	6.03(±0.57)
Gene diversity(H_e)	0.75	0.75	0.69	0.78	0.76(±0.02)	0.70	0.79	0.77(±0.03)

*1 Parental generation was a subset of wild fish, which were tentatively considered as unrelated fish
*2 Hypothetical hatchery strain consisting of the 83 G_1 offspring and 9 wild fish
mk-G_1 and *mk*-G_1^+ refer to a subgroup selected through mk_r procedures, and G_1-*rand* and G_1^+-*rand* are random samples selected through the Monte Carlo simulation with 100 iterations (see test for details)
For the random samples, the average of the A and H_e values are shown. Standard deviation of the measures is given in parenthesis.

benefit in retarding the loss of genetic diversity in terms of the allelic diversity and gene diversity, it gave however a poor performance to maintain the overall population fitness compared with other strategies including random mating and compensatory mating approach in which the highest mean kinship male in a population is mated with the lowest female (Santiago and Caballero, 1995; Caballero *et al.*, 1996), resulted from the inbreeding depression. Ballou and Lacy (1995) themselves state in their literature that the *mk* selection strategy would be accompanied by a risk of inbreeding especially in early generations, and they emphasize the necessity to preclude mating among highly related individuals. In this study, the eight mk_r-selected G_1 fish yielded 28 dyads of which three dyads were full-sibs, 10 were half-sibs, and the remaining 15 dyads were unrelateds. Although the sexes of the selected fish cannot be determined, it is very likely that the use of the subset of these fish to create the next generation would result in inbreeding to a greater or lesser extent. The breeding regimen should be thus designed so as to avoid possible sib mating, that is, unrelated pairs should be selected and mated to minimize inbreeding. When the mean r_{xy} value of unrelated pairs in the G_1 offspring (r_{xy}=-0.178) was applied as a cut-off value to discriminate the unrelated pairs from the other categories, one of the 15 unrelated pairs were still misclassified into half-sibs, and three of the 10 half-sibs, of which the smaller r_{xy} values than the cut-off value were yielded, were falsely assigned to the unrelated category; notwithstanding the kinship information derived from the r_{xy} statistic could greatly improve the efficiency in the avoidance of sib mating.

Summary

For the closed, or nearly closed hatchery production of Japanese flounder in the absence of pedigree information, the present author proposes several hatchery options as follows: first, genetically important individuals should be explored through the mk_r selection procedures. The mk_r selection would be more feasible in the Japanese flounder hatchery production rather than the original *mk* selection approach. Next, the kinships of the selected fish should be investigated by using the r_{xy} statistic as a guideline. The definition of the cut-off value to determine unrelated pairs depends on hatchery's motivations, it is although expected that applying more stringent cut-off values will achieve more accurate kinship-discrimination. Then the selected fish should be subdivided into multiple spawning tanks minimizing the risk of sib mating. These options may not necessary promise to stamp out the possibility of inbreeding, but will promise better outcomes than blind hatchery practices.

Chapter 7. Conclusions and perspectives

As described in Chapter 5, the minimum effective population size of 50 is widely accepted as a guideline value for the short-term hatchery production related to stock enhancement programs. It should however be noted that even if the reference figure could be achieved, stocking practice have an inherent risk to reduce the effective size of wild populations, especially when the contribution of stocked fish to reproduction in the natural populations is high (Chapter 5). In this regard, the notion of conservation genetics encounters a counterargument brought from the practical hatchery's scope: in the sense of conservation genetics, it may be preferable that the contribution of the stocked fish to the natural populations should be minimized to retain the effective size of wild populations at the current level; from the viewpoint of practical hatchery concept, however, the contribution of stocked fish to the natural populations should be maximized to increase the exploitable biomass. The ideas from the two sides are paradoxical, and it is considered, by the present author, that one of the compromise proposals is to increase the effective size of hatchery strains as much as possible.

Based on the results described in the previous chapters, the present author proposes here

several hatchery options to maximize the effective size of Japanese flounder hatchery strains, and to minimize the genetic impact of the stocking practice to the natural as follows: (1) captive broodstock to create new hatchery strains should be taken from the indigenous wild populations, and then the outcomes should be released back into the natural habitat from which the founders were derived. Because the wild Japanese flounder populations around Japan should not be managed as a single stock unit (see Chapter 3), and such efforts will encourage to preserve the currently detectable genetic stock units; (2) at the mating event, wild fish (unrelated in all likelihood) and spawning tanks should be prepared as many as possible, and the mating procedures should be carried out in separated tanks subdividing the candidate broodstock parents, rather than mating them in a single spawning tank. This mating design enables to minimize unexpected interactions among the candidates, which may allow a skewed family size dominated by a few broodstock parents (Chapter 5). The offspring pool into which the reproductive output derived from each tank are mixed will be available for stocking practices; (3) collection of fertilized eggs should be carried out for several days and on several times during the spawning season; (4) culling for particular traits should be avoided both during the rearing period and at the release event; (5) when the offspring pools to be stocked into the natural environment have to be created by using hatchery-based fish, the genetically important individuals in the strains of interest should be explored, and the kinships should be determined to avoid sib-mating as proposed in Chapter 6. A guideline for hatchery production, recommended by the present author, is summarized in Fig. 9.

To practically carry out the recommendations outlined above however may entail a trade-off between costs (labor, budget, and facility availability) and benefits in hatcheries; notwithstanding it should be emphasized that stock enhancement programs should not aim to yield a temporal harvestable surplus, but rather the perpetual exploitation of resources through the enhancement of natural stocks. Genetic considerations from the standpoint of conservation genetics are therefore essential for this ultimate task.

While, the genetic tagging of hatchery-based fish would provide the most efficient means to examine the genetic effects of stocking practice upon the natural gene pools. This study could not provide concrete evidence of the stocking effects to the natural populations, the present author however considers that it is an important task for future genetic studies related to stock enhancement, as there have been limited studies available to enhance our understanding of the genetic stocking effects to the wild populations in marine fish species (but see Perez-Enriquez *et al.*, 2001; Svasand *et al.*, 2000). A long-term monitoring of stocking effects can greatly enhance our understanding of the reproductive success of stocked fish; it can only be determined with the use of the heritable molecular markers. This will in turn contribute to give more insight into the genetic management strategy for the Japanese flounder resources in the natural environment.

Finally, the present author will continue to stress that stock enhancement practices should aim to encourage the natural productivity that will secure the exploitable resource biomass for the future without the need for artificial help, but should not be directed toward a temporary recovery of resource losses caused by a fishery-related management failures such as over-exploitation.

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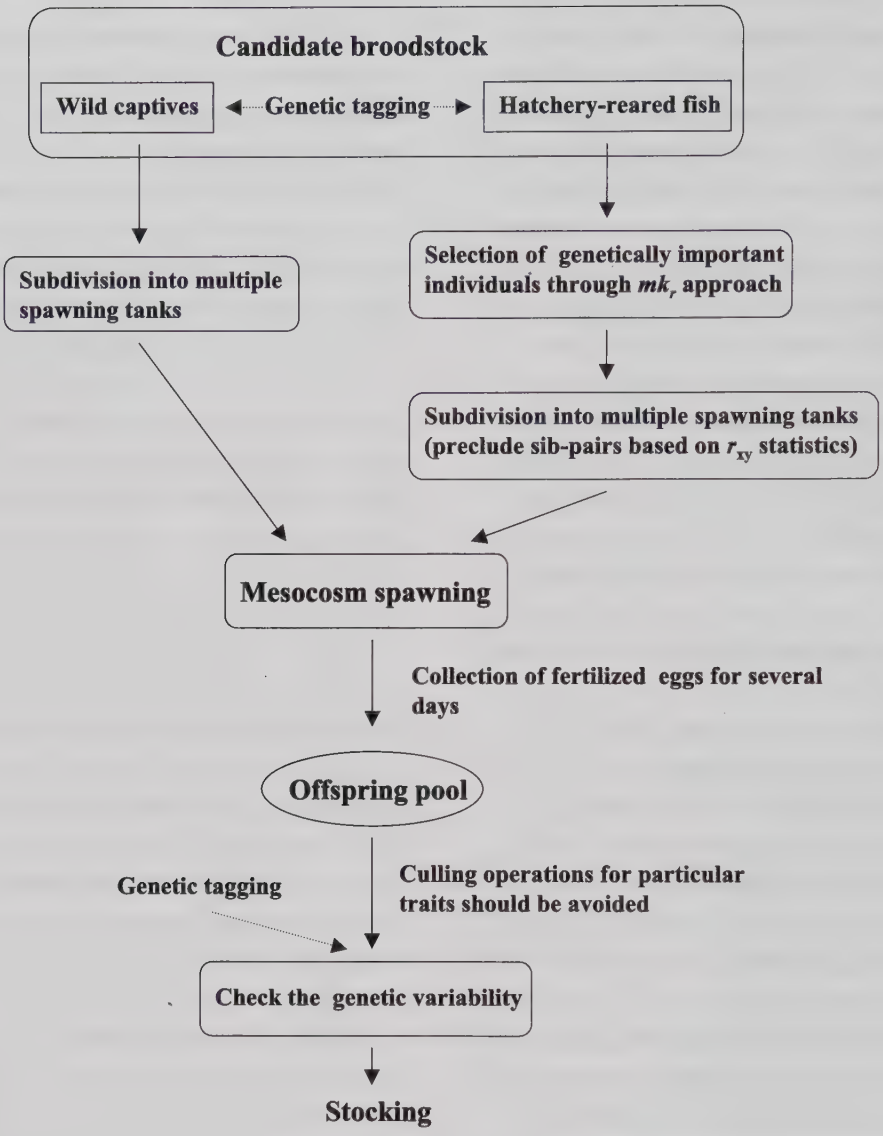
Candidate broodstock
Wild captives
Genetic tagging
Hatchery-reared fish
Subdivision into multiple spawning tanks
Selection of genetically important individuals through mk_r approach
Subdivision into multiple spawning tanks (preclude sib-pairs based on r_{xy} statistics)
Mesocosm spawning
Collection of fertilized eggs for several days
Offspring pool
Genetic tagging
Culling operations for particular traits should be avoided
Check the genetic variability
Stocking

Fig. 9. Hatchery management options to minimize loss of genetic variability in Japanese flounder hatchery strains

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Appendix 1. Allele frequency distributions of 11 microsatellites for the 7 wild Japanese flounder populations

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po1	162	0.330	0.330	0.406	0.424	0.389	0.420	0.423
	164	0.080	0.080	0.094	0.085	0.023	0.083	0.068
	166	0.000	0.020	0.000	0.000	0.023	0.007	0.011
	168	0.000	0.000	0.000	0.014	0.000	0.014	0.000
	170	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	172	0.010	0.040	0.036	0.049	0.057	0.049	0.023
	174	0.020	0.030	0.022	0.007	0.045	0.007	0.011
	176	0.020	0.030	0.015	0.007	0.011	0.007	0.000
	178	0.040	0.040	0.029	0.014	0.068	0.042	0.000
	180	0.050	0.030	0.036	0.056	0.057	0.028	0.045
	182	0.030	0.010	0.000	0.021	0.011	0.000	0.011
	184	0.040	0.040	0.044	0.056	0.034	0.035	0.080
	186	0.040	0.010	0.029	0.007	0.023	0.028	0.034
	188	0.120	0.040	0.051	0.085	0.068	0.042	0.034
	190	0.040	0.050	0.036	0.021	0.011	0.028	0.057
	192	0.010	0.050	0.036	0.042	0.034	0.035	0.023
	194	0.020	0.020	0.015	0.021	0.000	0.000	0.000
	196	0.030	0.050	0.029	0.014	0.034	0.021	0.057
	198	0.010	0.000	0.007	0.021	0.000	0.014	0.000
	200	0.000	0.040	0.007	0.014	0.034	0.049	0.023
	202	0.010	0.020	0.007	0.007	0.011	0.007	0.011
	204	0.030	0.020	0.007	0.000	0.023	0.007	0.000
	206	0.000	0.010	0.015	0.000	0.000	0.007	0.000
	208	0.000	0.000	0.007	0.000	0.000	0.000	0.011
	210	0.010	0.000	0.029	0.000	0.000	0.014	0.000
	212	0.000	0.010	0.007	0.007	0.000	0.000	0.011
	214	0.010	0.030	0.015	0.021	0.011	0.007	0.011
	216	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	218	0.010	0.000	0.007	0.000	0.000	0.014	0.023
	220	0.000	0.000	0.000	0.000	0.011	0.007	0.000
	222	0.000	0.000	0.000	0.000	0.011	0.000	0.011
	226	0.020	0.000	0.007	0.000	0.000	0.000	0.000
	228	0.010	0.000	0.000	0.000	0.000	0.007	0.011
	230	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	232	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	234	0.000	0.000	0.007	0.000	0.011	0.007	0.000
	264	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Po13	206	0.000	0.000	0.030	0.007	0.014	0.000	0.021
	208	0.100	0.100	0.160	0.109	0.111	0.103	0.099
	210	0.010	0.010	0.010	0.007	0.021	0.023	0.014
	212	0.160	0.160	0.070	0.101	0.049	0.068	0.106
	214	0.070	0.070	0.140	0.094	0.042	0.136	0.099
	216	0.090	0.090	0.150	0.174	0.118	0.218	0.136
	218	0.060	0.060	0.060	0.109	0.128	0.045	0.070
	220	0.050	0.050	0.050	0.036	0.042	0.011	0.035
	222	0.030	0.030	0.040	0.044	0.049	0.000	0.014
	224	0.050	0.050	0.040	0.029	0.069	0.080	0.049
	226	0.000	0.000	0.040	0.036	0.049	0.034	0.049
	228	0.030	0.030	0.010	0.015	0.063	0.045	0.042
	230	0.040	0.040	0.030	0.044	0.042	0.034	0.049
	232	0.070	0.070	0.010	0.007	0.035	0.045	0.021
	234	0.030	0.030	0.020	0.007	0.007	0.023	0.028
	236	0.040	0.040	0.020	0.022	0.021	0.034	0.028
	238	0.010	0.010	0.040	0.007	0.028	0.023	0.014
	240	0.020	0.020	0.010	0.029	0.021	0.011	0.014
	242	0.030	0.030	0.010	0.015	0.007	0.011	0.035
	244	0.030	0.030	0.020	0.029	0.007	0.011	0.021
	246	0.010	0.010	0.000	0.007	0.014	0.011	0.007
	248	0.010	0.010	0.010	0.007	0.014	0.011	0.000
	250	0.010	0.010	0.020	0.022	0.014	0.023	0.014
	252	0.020	0.020	0.000	0.007	0.000	0.000	0.014
	254	0.010	0.010	0.010	0.000	0.000	0.000	0.000
	256	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	258	0.000	0.000	0.000	0.007	0.014	0.000	0.000
	264	0.010	0.010	0.000	0.000	0.000	0.000	0.007
	266	0.000	0.000	0.000	0.000	0.007	0.000	0.000
	270	0.010	0.010	0.000	0.000	0.000	0.000	0.000
	274	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	278	0.000	0.000	0.000	0.022	0.000	0.000	0.007
	280	0.000	0.000	0.000	0.000	0.000	0.000	0.007
Po25A	201	0.050	0.010	0.015	0.014	0.034	0.014	0.023
	205	0.020	0.000	0.022	0.000	0.000	0.014	0.023
	209	0.020	0.020	0.022	0.021	0.023	0.021	0.023
	213	0.000	0.000	0.007	0.000	0.023	0.014	0.011
	217	0.070	0.122	0.116	0.127	0.068	0.127	0.125
	221	0.220	0.133	0.174	0.141	0.170	0.183	0.182
	225	0.220	0.235	0.152	0.254	0.205	0.141	0.238
	229	0.250	0.318	0.419	0.282	0.353	0.332	0.205

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po25A	233	0.030	0.051	0.015	0.049	0.045	0.035	0.034
	237	0.060	0.020	0.029	0.021	0.011	0.007	0.011
	241	0.000	0.010	0.000	0.014	0.023	0.014	0.034
	245	0.000	0.000	0.007	0.000	0.000	0.014	0.000
	249	0.000	0.020	0.000	0.000	0.011	0.007	0.000
	253	0.050	0.041	0.022	0.049	0.034	0.070	0.080
	257	0.000	0.010	0.000	0.014	0.000	0.007	0.000
	261	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	265	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	269	0.000	0.000	0.000	0.007	0.000	0.000	0.011
	305	0.000	0.000	0.000	0.007	0.000	0.000	0.000
Po26	141	0.170	0.210	0.250	0.275	0.273	0.188	0.291
	151	0.010	0.000	0.000	0.007	0.000	0.000	0.000
	153	0.010	0.000	0.015	0.000	0.023	0.007	0.012
	155	0.530	0.620	0.485	0.500	0.465	0.486	0.534
	157	0.010	0.010	0.015	0.021	0.034	0.090	0.023
	159	0.270	0.160	0.235	0.197	0.205	0.229	0.140
Po33	257	0.000	0.020	0.015	0.007	0.011	0.035	0.000
	261	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	263	0.600	0.592	0.529	0.500	0.547	0.562	0.559
	265	0.010	0.000	0.007	0.014	0.011	0.007	0.000
	278	0.000	0.031	0.051	0.028	0.011	0.014	0.000
	280	0.100	0.122	0.058	0.070	0.034	0.035	0.034
	282	0.000	0.010	0.029	0.014	0.011	0.000	0.011
	284	0.000	0.000	0.036	0.000	0.000	0.000	0.000
	286	0.080	0.082	0.130	0.085	0.091	0.083	0.011
	288	0.180	0.143	0.138	0.247	0.250	0.215	0.352
	290	0.020	0.000	0.007	0.014	0.000	0.021	0.011
	292	0.010	0.000	0.000	0.014	0.034	0.028	0.011
	294	0.000	0.000	0.000	0.007	0.000	0.000	0.000
Po35	279	0.000	0.010	0.000	0.007	0.000	0.000	0.011
	283	0.000	0.000	0.015	0.000	0.011	0.014	0.023
	285	0.390	0.400	0.421	0.389	0.365	0.368	0.457
	287	0.080	0.020	0.036	0.014	0.011	0.028	0.011
	289	0.190	0.150	0.167	0.176	0.193	0.190	0.136
	291	0.030	0.040	0.007	0.056	0.057	0.049	0.057
	293	0.000	0.020	0.007	0.028	0.011	0.028	0.000
	295	0.080	0.100	0.130	0.120	0.114	0.120	0.125
	297	0.030	0.040	0.022	0.035	0.023	0.035	0.034
	299	0.040	0.030	0.051	0.021	0.068	0.035	0.045
	301	0.040	0.030	0.029	0.014	0.023	0.035	0.045
	303	0.000	0.000	0.007	0.021	0.023	0.000	0.000
	305	0.030	0.010	0.015	0.014	0.023	0.028	0.023
	307	0.000	0.020	0.000	0.014	0.000	0.000	0.011
	309	0.010	0.000	0.007	0.000	0.000	0.000	0.000
	311	0.020	0.020	0.007	0.014	0.011	0.014	0.000
	313	0.010	0.010	0.007	0.000	0.000	0.007	0.000
	317	0.010	0.060	0.036	0.028	0.011	0.014	0.000
	319	0.010	0.020	0.022	0.021	0.045	0.000	0.000
	321	0.010	0.010	0.000	0.014	0.000	0.007	0.011
	323	0.000	0.010	0.007	0.007	0.011	0.014	0.011
	325	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	327	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	331	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	333	0.010	0.000	0.007	0.000	0.000	0.000	0.000
	343	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Po42	152	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	164	0.000	0.020	0.007	0.007	0.000	0.000	0.000
	166	0.020	0.020	0.000	0.007	0.023	0.021	0.000
	172	0.210	0.250	0.218	0.199	0.139	0.149	0.231
	174	0.190	0.070	0.116	0.120	0.068	0.085	0.091
	176	0.030	0.050	0.101	0.106	0.114	0.099	0.091
	178	0.080	0.070	0.101	0.127	0.080	0.141	0.136
	180	0.070	0.040	0.058	0.063	0.068	0.078	0.068
	182	0.080	0.100	0.044	0.042	0.034	0.042	0.068
	184	0.010	0.030	0.058	0.056	0.045	0.028	0.011
	186	0.050	0.010	0.065	0.014	0.045	0.035	0.057
	188	0.050	0.030	0.015	0.021	0.068	0.028	0.023
	190	0.040	0.030	0.015	0.014	0.023	0.035	0.011
	192	0.010	0.010	0.036	0.014	0.023	0.035	0.034
	194	0.000	0.030	0.029	0.042	0.011	0.028	0.000
	196	0.020	0.000	0.015	0.028	0.000	0.007	0.011
	198	0.010	0.040	0.007	0.014	0.034	0.021	0.057
	200	0.010	0.030	0.007	0.007	0.023	0.042	0.011
	202	0.020	0.020	0.029	0.021	0.045	0.021	0.011
	204	0.010	0.030	0.007	0.028	0.011	0.007	0.023

Appendix 1. (continued)

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po42	206	0.000	0.000	0.000	0.021	0.045	0.014	0.000
	208	0.020	0.030	0.036	0.028	0.011	0.028	0.011
	210	0.000	0.010	0.007	0.000	0.011	0.000	0.011
	212	0.030	0.010	0.000	0.000	0.023	0.000	0.000
	214	0.010	0.010	0.000	0.000	0.000	0.007	0.011
	216	0.000	0.020	0.015	0.000	0.023	0.007	0.000
	218	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	220	0.000	0.000	0.007	0.007	0.000	0.007	0.000
	222	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	224	0.010	0.010	0.007	0.007	0.000	0.007	0.011
	226	0.000	0.010	0.000	0.000	0.011	0.007	0.011
	230	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	232	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	242	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	250	0.000	0.010	0.000	0.000	0.000	0.007	0.000
	254	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	270	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Po48	118	0.000	0.010	0.000	0.007	0.000	0.000	0.000
	120	0.010	0.010	0.000	0.014	0.011	0.021	0.023
	122	0.010	0.000	0.000	0.000	0.011	0.007	0.000
	126	0.640	0.740	0.696	0.740	0.740	0.693	0.659
	128	0.000	0.010	0.007	0.014	0.000	0.021	0.000
	130	0.300	0.180	0.239	0.190	0.216	0.188	0.239
	134	0.000	0.020	0.022	0.007	0.011	0.014	0.057
	138	0.030	0.030	0.022	0.014	0.000	0.035	0.011
	142	0.000	0.000	0.007	0.000	0.000	0.007	0.000
	146	0.010	0.000	0.007	0.014	0.011	0.014	0.000
	150	0.000	0.000	0.000	0.000	0.000	0.000	0.011
Po52	153	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	155	0.800	0.760	0.660	0.643	0.774	0.819	0.716
	157	0.010	0.010	0.015	0.014	0.011	0.021	0.011
	159	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	161	0.060	0.160	0.246	0.286	0.136	0.104	0.125
	163	0.130	0.070	0.079	0.057	0.057	0.056	0.148
Po56	139	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	141	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	147	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	151	0.000	0.020	0.000	0.000	0.000	0.000	0.000
	153	0.040	0.000	0.022	0.014	0.011	0.007	0.000
	155	0.000	0.000	0.000	0.000	0.011	0.007	0.000
	157	0.010	0.070	0.015	0.000	0.023	0.028	0.000
	159	0.150	0.060	0.080	0.035	0.116	0.070	0.114
	161	0.050	0.030	0.051	0.085	0.045	0.056	0.068
	163	0.050	0.060	0.051	0.007	0.011	0.000	0.011
	165	0.090	0.120	0.101	0.113	0.068	0.092	0.045
	167	0.080	0.050	0.029	0.028	0.034	0.035	0.045
	169	0.030	0.010	0.015	0.014	0.034	0.042	0.011
	171	0.020	0.010	0.044	0.021	0.034	0.028	0.045
	173	0.100	0.030	0.073	0.085	0.102	0.099	0.128
	175	0.040	0.110	0.105	0.120	0.057	0.149	0.091
	177	0.060	0.120	0.065	0.056	0.057	0.085	0.102
	179	0.060	0.040	0.080	0.128	0.068	0.113	0.102
	181	0.040	0.040	0.051	0.042	0.057	0.021	0.034
	183	0.060	0.080	0.065	0.035	0.045	0.028	0.045
	185	0.000	0.030	0.022	0.042	0.023	0.021	0.023
	187	0.000	0.020	0.029	0.000	0.023	0.014	0.011
	189	0.010	0.000	0.007	0.000	0.023	0.014	0.023
	191	0.010	0.010	0.015	0.056	0.023	0.000	0.023
	193	0.010	0.000	0.000	0.014	0.023	0.007	0.000
	195	0.030	0.040	0.007	0.028	0.011	0.014	0.023
	197	0.010	0.020	0.022	0.014	0.011	0.035	0.011
	199	0.040	0.010	0.007	0.007	0.034	0.014	0.023
	201	0.000	0.020	0.015	0.035	0.011	0.021	0.011
	203	0.000	0.000	0.015	0.000	0.023	0.000	0.011
	205	0.010	0.000	0.007	0.007	0.000	0.000	0.000
	207	0.000	0.000	0.000	0.007	0.011	0.000	0.000
Po91	140	0.000	0.000	0.000	0.000	0.000	0.000	0.023
	142	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	144	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	146	0.070	0.060	0.058	0.042	0.080	0.028	0.034
	148	0.090	0.070	0.094	0.085	0.068	0.056	0.080
	150	0.050	0.050	0.029	0.063	0.034	0.049	0.080
	152	0.000	0.010	0.007	0.000	0.000	0.021	0.023
	154	0.040	0.020	0.036	0.028	0.045	0.049	0.023

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po91	158	0.060	0.060	0.094	0.099	0.162	0.104	0.068
	160	0.100	0.200	0.115	0.099	0.034	0.069	0.080
	162	0.110	0.060	0.094	0.085	0.057	0.056	0.057
	164	0.020	0.070	0.029	0.056	0.045	0.090	0.068
	166	0.100	0.020	0.015	0.035	0.011	0.007	0.000
	168	0.020	0.000	0.007	0.014	0.034	0.021	0.000
	170	0.000	0.030	0.007	0.014	0.045	0.049	0.023
	172	0.030	0.020	0.000	0.028	0.023	0.021	0.023
	174	0.010	0.010	0.022	0.042	0.000	0.021	0.011
	176	0.030	0.020	0.036	0.021	0.000	0.028	0.011
	178	0.000	0.030	0.000	0.000	0.011	0.007	0.045
	180	0.000	0.020	0.015	0.007	0.034	0.000	0.023
	182	0.000	0.010	0.000	0.007	0.000	0.007	0.000
	184	0.010	0.010	0.029	0.007	0.011	0.000	0.000
	186	0.000	0.000	0.007	0.000	0.000	0.007	0.034
	188	0.010	0.010	0.000	0.000	0.000	0.021	0.011
	190	0.020	0.010	0.015	0.007	0.023	0.007	0.011
	192	0.000	0.000	0.007	0.014	0.011	0.007	0.023
	194	0.010	0.000	0.015	0.021	0.034	0.035	0.023
	196	0.000	0.010	0.007	0.000	0.023	0.014	0.011
	198	0.000	0.000	0.015	0.000	0.023	0.000	0.011
	200	0.000	0.000	0.029	0.028	0.034	0.007	0.023
	202	0.020	0.050	0.022	0.021	0.011	0.007	0.011
	204	0.000	0.010	0.000	0.000	0.000	0.021	0.011
	206	0.000	0.000	0.015	0.021	0.011	0.000	0.011
	208	0.000	0.000	0.007	0.000	0.011	0.014	0.000
	210	0.000	0.000	0.015	0.014	0.023	0.000	0.011
	212	0.020	0.000	0.000	0.000	0.000	0.007	0.000
	214	0.010	0.000	0.000	0.007	0.011	0.007	0.000
	216	0.000	0.000	0.000	0.000	0.000	0.014	0.000
	218	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	222	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	226	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	230	0.000	0.000	0.000	0.014	0.000	0.000	0.000
	232	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	234	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	236	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	238	0.000	0.000	0.015	0.000	0.000	0.000	0.000
	240	0.010	0.000	0.007	0.007	0.000	0.000	0.000
	242	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	244	0.000	0.000	0.007	0.000	0.000	0.000	0.011
	246	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	264	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	268	0.000	0.000	0.000	0.000	0.000	0.007	0.000

Appendix 2. Allele frequency distributions of 11 microsatellites for the 3 hatchery strains of Japanese flounder

Locus	Alleles	Sample names		
		HY	HF	HC
Po1	162	0.550	0.810	0.390
	164	0.030	0.100	0.045
	172	0.070	0.000	0.030
	174	0.040	0.030	0.005
	176	0.005	0.000	0.000
	178	0.000	0.000	0.115
	180	0.030	0.010	0.120
	184	0.140	0.000	0.045
	186	0.005	0.000	0.000
	188	0.075	0.050	0.000
	192	0.030	0.000	0.165
	196	0.000	0.000	0.010
	200	0.000	0.000	0.035
	210	0.005	0.000	0.030
	222	0.020	0.000	0.010
Po13	208	0.165	0.000	0.210
	212	0.050	0.225	0.005
	214	0.060	0.040	0.060
	216	0.305	0.600	0.100
	218	0.130	0.000	0.100
	220	0.015	0.000	0.035
	224	0.010	0.005	0.005
	226	0.080	0.005	0.005
	228	0.005	0.000	0.015
	230	0.000	0.015	0.120
	232	0.020	0.000	0.005
	234	0.010	0.000	0.075
	236	0.030	0.000	0.000
	240	0.070	0.000	0.000
	244	0.000	0.025	0.065
	246	0.000	0.085	0.000
	248	0.000	0.000	0.095
	250	0.045	0.000	0.000
	256	0.000	0.000	0.105
	274	0.005	0.000	0.000
Po25A	201	0.005	0.000	0.010
	209	0.015	0.000	0.060
	213	0.090	0.000	0.000
	217	0.145	0.000	0.360
	221	0.135	0.135	0.040
	225	0.200	0.285	0.215
	229	0.250	0.300	0.220
	233	0.015	0.000	0.095
	237	0.030	0.000	0.000
	241	0.050	0.000	0.000
	245	0.000	0.120	0.000
	249	0.055	0.000	0.000
	253	0.005	0.125	0.000
	257	0.005	0.035	0.000
Po26	141	0.335	0.205	0.160
	155	0.420	0.365	0.645
	159	0.245	0.430	0.195
Po33	257	0.025	0.010	0.000
	263	0.445	0.470	0.450
	278	0.065	0.000	0.000
	280	0.205	0.150	0.000
	282	0.000	0.035	0.010
	286	0.090	0.025	0.045
	288	0.160	0.075	0.475
	290	0.000	0.235	0.020
	292	0.010	0.000	0.000
Po35	285	0.215	0.445	0.545
	287	0.035	0.150	0.005
	289	0.280	0.105	0.055
	291	0.000	0.080	0.105
	293	0.105	0.000	0.020
	295	0.110	0.000	0.155
	297	0.000	0.000	0.010
	299	0.030	0.040	0.000
	301	0.080	0.030	0.015
	305	0.010	0.000	0.040
	309	0.005	0.000	0.000
	311	0.010	0.000	0.000
	313	0.000	0.110	0.000
	317	0.000	0.000	0.045
	319	0.115	0.000	0.000
	321	0.000	0.040	0.000
	323	0.000	0.000	0.005
	329	0.005	0.000	0.000
Po42	166	0.015	0.060	0.000
	168	0.000	0.000	0.005
	172	0.335	0.000	0.275
	174	0.120	0.135	0.050
	176	0.035	0.465	0.010
	178	0.020	0.000	0.100
	180	0.030	0.075	0.000
	182	0.070	0.000	0.025
	184	0.035	0.000	0.090
	188	0.120	0.000	0.135
	190	0.065	0.000	0.065
	192	0.010	0.000	0.000
	196	0.010	0.000	0.100
	200	0.055	0.185	0.000
	202	0.000	0.000	0.015
	204	0.000	0.000	0.085
	206	0.010	0.000	0.030
	212	0.000	0.000	0.010
	214	0.000	0.050	0.005
	218	0.000	0.030	0.000
	248	0.070	0.000	0.000
Po48	120	0.000	0.000	0.005
	126	0.610	0.740	0.510
	128	0.000	0.000	0.045
	130	0.280	0.260	0.385
	134	0.005	0.000	0.020
	138	0.050	0.000	0.000
	146	0.000	0.000	0.035
	154	0.055	0.000	0.000
Po52	155	0.970	0.975	0.895
	161	0.000	0.025	0.070
	163	0.025	0.000	0.035
	165	0.005	0.000	0.000
Po56	147	0.005	0.000	0.000
	153	0.100	0.000	0.045
	157	0.010	0.000	0.085
	159	0.060	0.155	0.035
	161	0.000	0.000	0.070
	163	0.020	0.000	0.000
	165	0.190	0.040	0.040
	167	0.000	0.025	0.080
	169	0.005	0.000	0.005
	171	0.000	0.000	0.015
Po56	173	0.140	0.315	0.160
	175	0.110	0.000	0.090
	177	0.185	0.000	0.005
	179	0.055	0.055	0.025
	181	0.000	0.000	0.005
	183	0.020	0.385	0.075
	185	0.000	0.000	0.020
	189	0.055	0.000	0.000
	191	0.030	0.000	0.000
	193	0.000	0.000	0.115
	197	0.005	0.025	0.035
	201	0.010	0.000	0.095
Po91	146	0.100	0.040	0.060
	148	0.140	0.000	0.105
	150	0.070	0.000	0.040
	154	0.045	0.025	0.000
	156	0.265	0.175	0.275
	158	0.085	0.055	0.000
	160	0.100	0.410	0.120
	162	0.065	0.010	0.015
	164	0.005	0.000	0.020
	166	0.005	0.000	0.000
	168	0.000	0.000	0.045
	172	0.055	0.100	0.065
	176	0.000	0.000	0.070
	184	0.015	0.000	0.035
	188	0.000	0.140	0.070
	190	0.000	0.000	0.015
	192	0.000	0.000	0.030
	196	0.000	0.000	0.035
	200	0.015	0.000	0.000
	204	0.000	0.010	0.000
	208	0.005	0.000	0.000
	210	0.010	0.000	0.000
	224	0.015	0.000	0.000
	228	0.000	0.035	0.000
	232	0.005	0.000	0.000

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和文要旨

塩化トリブチルスズ（TBTCl）の組織器官への生物濃縮の成熟に伴った変化

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塩化トリブチルスズ化合物のマミチョグ（*Fundulus heteroclitus*）の全魚体及び各器官への生物濃縮や排泄の性及び成熟過程による変動が長期間の飼育実験により調べられた。不応期における全魚体の生物濃縮係数（BCF）は雄で9,100，また，雌で9,200であり，BCF及び体内分布ともにマミチョグの性による差は認められなかった。一方，TBTClは成熟期マミチョグの精巣に卵巣中濃度の約7倍もの高濃度に蓄積され，TBTClが精巣の発達過程を著しく阻害することが生物濃縮の観点からも示唆された。

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成熟期マミチョグの長期飼育試験による4-*tert*-オクチルフェノールの生物濃縮と体内分布

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エストロジェン様内分泌かく乱物質の一つである4-*tert*-オクチルフェノール（OP）の成熟期マミチョグによる生物濃縮，体内分布及び排泄を飼育実験により調べた。雌雄のマミチョグの生物濃縮係数は，それぞれ，34±11及び23±5.1であり，コイ及びヒメダカより小さく，また，性による差異は認められなかった。組織・器官のOP濃度は雌雄で大差なかった。一方，卵巣に蓄積されたOPは他の器官に比べて排泄が遅く，OPは卵への蓄積を通して次世代仔魚の初期発生を阻害することが示唆された。

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水産生物遺伝資源保存事業海洋微生物部門ベースコレクションの生物性状

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（独）水産総合研究センターが実施する遺伝資源保存事業において，微生物サブバンクとして位置づけられている中央水産研究所応用微生物研究室に収集されてある微生物菌株を整理し，2003年3月時点で50株をベースコレクションとして登録した。これら50菌株について，分譲利用される際の一助となるべく性状分析を行い，既知の遺伝子塩基配列情報と合わせて本稿にまとめて記載した。主な試験項目は，基礎性状試験，NF-18試験（グラム陰性菌），EB-20試験（グラム陽性菌），API 50CH試験およびAPI zym 20試験である。さらに細菌株については16S rRNA遺伝子（16S rDNA），酵母株については18S rRNA遺伝子（18S rDNA）の部分塩基配列をそれぞれ決定し，これらの結果を基に同定を行った。

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ヒラメ栽培漁業における親魚の遺伝的管理に関する研究

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近年の生物多様性の保全に対する関心の高まりとともに，種内に維持されている遺伝的多様性の重要性が認識されるようになってきた。遺伝的多様性の損失は，種が潜在的に持っている再生産力，環境変動への適応性，疾病に対する抵抗力等の低下を導くおそれがある。栽培漁業における種苗生産では，少数の天然魚または継代飼育個体を親魚として用いることが多いため，一般的に種苗の遺伝的多様性は低下している。遺伝学的観点から見た栽培漁業の問題点として，人工種苗の大量放流により，天然魚群の遺伝的多様性が低下し，天然魚が潜在的に持つ生産力が減少すること，また育種素材としての遺伝子資源の損失を招くリスクがあることが挙げられる。ヒラメ *Paralichthys olivaceus*は，重要な増養殖対象種となっており，全国の沿岸域で栽培漁業が展開されている。これまでのヒラメ人工種苗生産では，遺伝的多様性の保全に対する関心は高いとは言えず，疾病・斃死防除による効率的大量生産に焦点が当てられてきた。今後の種苗生産では，量的な到達目標を設定するだけでなく，種苗の遺伝的多様性を高く保ち，天然資源への遺伝的影響を最小限にするという，遺伝的な質の向上を目指していく必要がある。本研究では，ヒラメのDNAマーカーを開発し，それを用いて，天然魚と放流種苗の保有する遺伝的多様性・特徴を把握した上で，人工種苗の遺伝的多様性の損失を軽減しうる種苗生産法について考察した。本論文は全7章で構成される。第1章で研究の背景と目的について述べ，第2章では，ヒラメのマイクロサテライトDNA 座を単離し，DNAマーカーとしての利用性・妥当性を検証した。第3章では，マイクロサテライトDNA解析とミトコンドリアDNA調節領域シーケンス解析により，天然ヒラメ集団の持つ遺伝的多様性を見積もるとともに，その遺伝的集団管理単位を把握するため，地域間の遺伝的異質性を調べた。第4章で，実際のヒラメ人工種苗と天然魚集団の遺伝的多様性を比

較し，第5章では，マイクロサテライトDNAマーカーをヒラメ人工種苗の親子判別技術に応用して，本手法の人工種苗の遺伝的多様性モニタリングにおける有効性を実証した。第6章では，マイクロサテライトDNAマーカーを用いて人工種苗の血縁関係の推定を試み，それを指標とした遺伝的多様性の損失を軽減するための種苗生産法について考察した。第7章では，今後のヒラメ種苗生産のあり方について言及した。

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